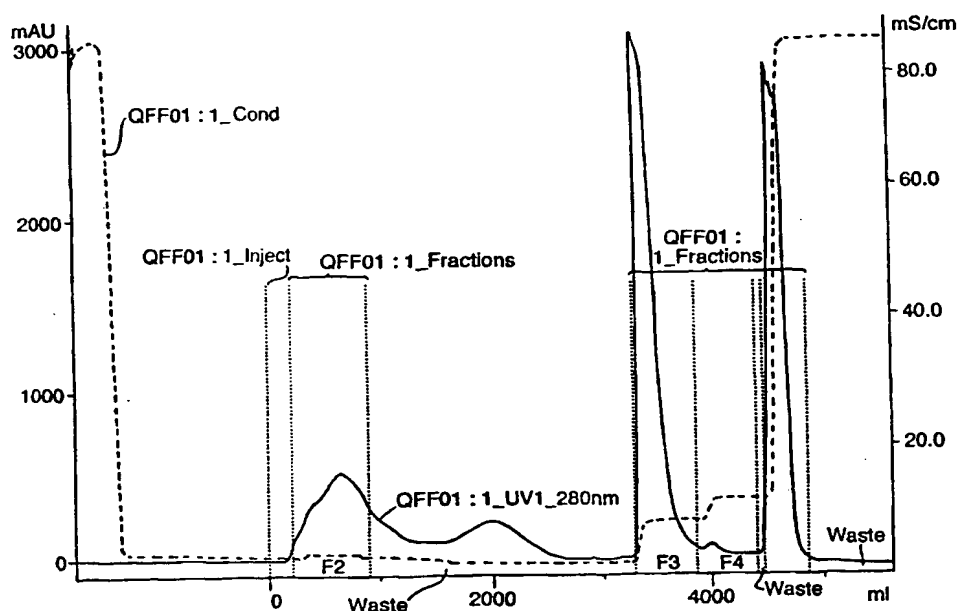




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(54) Title: PURIFICATION OF HUMAN ACID  $\alpha$ -GLUCOSIDASE

## (57) Abstract

The invention provides methods of purifying human acid  $\alpha$ -glucosidase, particularly from the milk of transgenic animals. The methods employ two chromatography steps. The first step is usually anion exchange chromatography and the second step is hydrophobic interaction chromatography. The purification procedure readily generates human  $\alpha$ -glucosidase in at least 99 % w/w purity. Also provided are pharmaceutical compositions and methods for using purified human acid  $\alpha$ -glucosidase in treatment of patients with Pompe's disease.

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PURIFICATION OF HUMAN ACID  $\alpha$ -GLUCOSIDASE

## 5 CROSS-REFERENCE TO RELATED APPLICATION

USSN 08/700,760 filed July 19, 1996 is directed to related subject matter and is incorporated by reference in its entirety for all purposes.

## 10 TECHNICAL FIELD

The invention resides in technical fields of protein chemistry and medicine.

## BACKGROUND

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Acid  $\alpha$ -glucosidase (acid maltase) is a enzyme with an essential function in the lysosomal degradation of glycogen to glucose [Rosenfeld, E.L. (1975) *Pathol. Biol.* 23. 71-84]. Pathological conditions occur with complete enzyme deficiency or when the functional enzyme is present in low amounts. Massive  
20 accumulation of glycogen is observed in the lysosomes, disrupting cellular function [reviewed by Hirschhorn, R. (1995) in *The Metabolic and Molecular Basis of Inherited Disease*, eds. Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D. (McGraw-Hill New York), 7<sup>th</sup> Ed., Vol. 2, pp. 2443-2464]. Human acid  $\alpha$ -glucosidase was discovered in 1963 as the primary defect in  
25 Glycogenesis Type II (Pompe's disease) [Hers, H.G. (1963) *Biochem. J.* 86, 11-16; Hers, H.G. and De Barsey, Th. (1973) in *Lysosomes and Storage Diseases* (Hers, H.G., and Van Hoof, F., eds) Pp. 197-216].

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Until then Glycogenesis Type II was known as an inherited, generalized, glycogen storage disease with fatal outcome in the first two years of life. It was later realized that the disease can occur in milder form in juvenile, and

in late-onset or adult forms, in which the major clinical symptoms are skeletal muscle weakness and myocardial myopathy [Howell, R.R. and Williams, J.C. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury et al, eds) Pp. 141-166, McGraw-Hill Book Co., New York]. Several clinical phenotypes have been observed [reviewed by Hirschhorn, R. (1995) in *The Metabolic and molecular bases of inherited disease* (Scriver et al. Eds) Pp. 2443-2464], and some are associated with identified mutations within the human acid  $\alpha$ -glucosidase gene (reviewed by Reuser et al, Suppl. 3 (1995) *Muscle and Nerve*, Pp.S61-S69].

Human acid  $\alpha$ -glucosidase is produced in the cell as a 110 kD precursor form. The seven potential N-linked glycosylation sites are probably all used (Hermans et al, (1993) *Biochem. J.* 289, 681-686). The carbohydrate chains are supposed to be of the high mannose type. In the Golgi stack specific mannose residues attached to the precursor are phosphorylated, yielding mannose-6-P. These residues are recognized by the mannose-6-phosphate receptor, which targets proteins to the lysosomes (reviewed by Von Figura & Hasilik, (1986) *Ann. Rev. Biochem.* 55, 167-193; reviewed by Kornfeld, S., (1992) *Ann. Rev. Biochem.* 61, 307-330). Within the lysosomes, N- and C-terminal processing finally leads, via a 95 kD human acid  $\alpha$ -glucosidase intermediate, to the mature 70 and 76 kD enzymes. The mature enzymes are active in the breakdown of glycogen to glucose (Hasilik & Neufeld, *J. Biol. Chem.* (1980) 255, 4937-4946; Hasilik & Neufeld, *J. Biol. Chem.* (1980) 255, 4946-4950; Martiniuk et al, *Arch. Biochem. Biophys.* (1984) 231, 454-460; Reuser et al, *J. Biol. Chem.* (1985) 260, 8336-8341; Reuser et al, *J. Clin. Invest.* (1987) 79, 1689-1699).

In glycogenesis type II, the lower (or absence of) enzyme activity could be due to many factors, like no or partial mRNA levels, no synthesis of human acid  $\alpha$ -glucosidase precursor, or no processing to mature enzyme. Also

mature enzyme can be produced, but with lower or no activity (reviewed by Hirschhorn, R. (1995) in *The Metabolic and molecular bases of inherited disease* (Scriver et al. Eds) Pp. 2443-2464; reviewed by Reuser et al, *Muscle & Nerve*, Suppl. 3 (1995) Pp S61-S69).

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Since the discovery of this and other lysosomal storage diseases, enzyme replacement therapy for Pompe patients has been attempted as a possible treatment. However, the trials were not successful. They were limited in the duration of treatment, and in the amount of enzyme administered. Moreover, either non-human acid  $\alpha$ -glucosidase from *Aspergillus niger*, giving immunological reactions, or "low-uptake" (nonphosphorylated) enzyme from human placenta were used [Baudhuin et al, (1964) *Lab. Invest.* 13, 1139-1152; Lauer et al, (1968) *Pediatrics* 42, p. 672; De Barsey et al (1973) In *Enzyme Therapy in Genetic Diseases* (Eds. Desnick, Bernlohr, Krivit) Williams & Wilkins, Baltimore, Pp. 184-190].

Since the isolation of the gene [Hoefsloot et al (1988) *EMBO J.* 7, 1697-1704; Hoefsloot et al (1990) *Biochem. J.* 272, 493-497; Martiniuk et al (1990) *DNA Cell Biol.* 9, 85; Martiniuk et al (1991) *DNA Cell Biol.* 10, 283] expression of recombinant human acid  $\alpha$ -glucosidase has been reported. Recombinant human acid  $\alpha$ -glucosidase made in baculovirus-infected insect cells was active but not taken up efficiently by Pompe patient's fibroblasts [Martiniuk et al. (1992) *DNA Cell Biol.* 11, 701-706]. Fuller et al. [(1995) *Eur. J. Biochem.* 234, 903-909] and Van Hove et al [(1996) *Proc. Natl. Acad. Sci. USA.* 93, 65-70], have reported expression in the medium of human precursor acid  $\alpha$ -glucosidase of cDNA-transfected Chinese hamster ovary cells.

Acid  $\alpha$ -glucosidase has been purified from a variety of tissues [see review of Hirschhorn, R. (1995) in *The Metabolic and molecular bases of inherited disease* (Scriver et al. Eds) Pp. 2443-2464]. Many reported procedures are

based on two properties of the enzyme: (1) the enzyme is N-glycosylated (predominantly high mannose), so the lectin Concanavalin A coupled to a matrix like Sepharose can be used; and (2) the enzyme has affinity for (1,4  $\alpha$  and (1,6  $\alpha$ -glycosidic linkages, so the enzyme under certain conditions is  
5 retarded on a gel-filtration matrices like Sephadex (contains (1,6 linkages) resulting in an affinity type of purification. A number of examples of methods to purify acid  $\alpha$ -glucosidase from various tissues are given below.

Jeffrey et al [(1970) *Biochem. J.* 9, 1403-1415] report the purification of the  
10 enzyme from rat liver. After homogenization and centrifugation, the lysosomes were disrupted, and the supernatant, obtained after high-speed centrifugation, was precipitated with 42% ammonium sulphate. The pellet, was resuspended, dialyzed, and loaded on a Sephadex G-100 column. The  $\alpha$ -glucosidase fractions from the column were loaded on a weak anion  
15 exchange column, and bound enzyme was eluted with 250 mM KCl. The purified enzyme was lyophilized.

Palmer [(1971) *Biochem. J.* 124, 701-711] report the purification of acid  $\alpha$ -glucosidase from rabbit muscle. Minced rabbit muscle was washed to  
20 remove blood components, homogenized, freeze/thawed, centrifuged, and the precipitate was re-extracted. The combined supernatant were acidified, again centrifuged, and the supernatant was first precipitated with 30% ammonium sulphate. The supernatant was precipitated again, now with 60% ammonium sulphate. The pellet was dissolved in low salt buffer and  
25 dialyzed. After freeze/drying, the enzyme was loaded on a Sephadex G-100 column for further purification.

Schram et al [(1979) *Biochim. Biophys. Acta* 567, 370-383] report purification of acid  $\alpha$ -glucosidase from human liver. After homogenization  
30 and high-speed centrifugation, the supernatant was loaded on a concanavalin A column. Bound enzyme was eluted with 1 M

methyl-glucoside, concentrated, dialyzed, and loaded on a S-200 gel-filtration column to obtain purified enzyme.

5 Martiniuk et al [(1984) *Arch. Biochem. Biophys.* 231, 454] report the purification of acid  $\alpha$ -glucosidase from human placenta. After homogenization and centrifugation, the supernatant was loaded on a CM-Sepharose column, essentially to remove hemoglobin. After centrifugation at 27,000 g (15 min), the homogenate was precipitated with 80% ammonium sulphate, centrifuged, and the supernatant was dialyzed,  
10 again centrifuged and loaded on a Sephadex G-100 column to obtain purified enzyme.

Reuser et al [(1985) *J. Biol. Chem.* 260, 8336-8341] report the purification of acid  $\alpha$ -glucosidase from human placenta. After homogenization and  
15 centrifugation, the supernatant was filtered, and loaded on a Concanavalin A Sepharose column. Bound enzyme was eluted with 1 M methyl glucoside, concentrated, dialyzed, and again concentrated by ultrafiltration before loading on a Sephadex G-200 column. The retarded enzyme was collected from the column and stored frozen.

20 Lin et al [(1992) *Hybridoma* 11, 493] report the purification of acid  $\alpha$ -glucosidase from human urine. The urine was concentrated by ultrafiltration, followed by Concanavalin A column chromatography. Eluted enzyme was precipitated with 80% ammonium sulphate. The pellet was redissolved in  
25 PBS, and loaded on a Sephadex G-100 column. The enzyme eluting from the column was again precipitated with 80% ammonium sulphate, and the redissolved pellet was loaded on a DEAE anion column. Bound enzyme was eluted with 0.1 M NaCl buffer. A 70 kD enzyme was visualized on SDS-PAGE.

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Fuller et al [(1995) *Eur. J. Biochem.* 234, 903-909] report the purification of recombinant human acid  $\alpha$ -glucosidase from the medium of cDNA-transfected Chinese hamster ovary cells. After clarifying the culture medium by low-speed centrifugation, the pH is adjusted to 6.6, and the medium was run over a Concanavalin A Sepharose column. Recombinant human acid  $\alpha$ -glucosidase is eluted with 1 M methyl-glucoside buffer and concentrated by ultrafiltration. The concentrate is loaded on a Sephadex G-100 column and fractionated at a low flow rate to obtain purified human acid  $\alpha$ -glucosidase.

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Van Hove et al [(1996) *Proc. Natl. Acad. Sci. USA.* 93, 65-70] report the isolation of recombinant human acid ( $\alpha$ -glucosidase produced in the medium of transfected CHO cells using similar techniques.

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Van Hove et al [(1997) *Biochem. Mol. Biol. Int.* 43, 613-623] report the isolation of recombinant human acid  $\alpha$ -glucosidase produced in the medium of transfected CHO cells using the following techniques: after addition of a suitable binding buffer, the medium was loaded on a Concanavalin A column.  $\alpha$ -glucosidase was eluted with a 1 M methyl glucoside buffer.

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Ammonium-sulphate was added, and the sample was loaded on a Phenyl Sepharose HP column. The column was washed, and contaminating proteins were eluted with a gradient of 25-45% elution buffer (20 mM acetate pH 5.3).

25

Subsequently,  $\alpha$ -glucosidase was eluted with a gradient to 100% elution buffer. The enzyme containing fractions were concentrated by ultrafiltration (Amicon stirred bar cell, YM30 membrane), and the enzyme was applied to a Superdex 200 prep grade column. Enzyme was eluted isocratically with 25 mM NaCl, 20 mM acetate buffer pH 4.6 at a low flow rate of 2.5 ml/min. Enzyme containing solutions were pooled, dia-filtered in the stirred bar cell against a 10 mM NaCl, 25 mM histidine pH 5.5. After loading the sample on

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a Source Q column, the column was washed with 2% elution buffer (500 mM



NaCl, 25 mM histidine pH 5.5) and bound acid  $\alpha$ -glucosidase was eluted with a gradient of 24% elution buffer.

5 Several features of the above methods are not ideal for achieving large scale purification of human acid  $\alpha$ -glucosidase for therapeutic use. Use of concanavalin A is disadvantageous because it is mitogenic to human lymphocytes and can also give rise to allergy problems [Mody et al (1995) *J. Pharmacol. Toxicol. Methods* 33, 1-10]. Processing of fractions containing acid  $\alpha$ -glucosidase on gel filtration columns, i.e. Sephadex, is too time  
10 consuming and cumbersome for large-scale operation.

#### SUMMARY OF THE CLAIMED INVENTION

15 Accordingly, in one aspect the present invention provides a method of purifying human acid  $\alpha$ -glucosidase comprising:

(a) applying a sample containing human acid  $\alpha$ -glucosidase and  
contaminating proteins to an anion exchange or an affinity column under  
20 conditions in which the  $\alpha$ -glucosidase binds to the column;

(b) collecting an eluate enriched in  $\alpha$ -glucosidase from the anion exchange or affinity column;

25 (c) applying the eluate to

(i) a hydrophobic interaction column under conditions in which  $\alpha$ -glucosidase binds to the column and then collecting a further eluate further enriched in  $\alpha$ -glucosidase, or

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- (ii) contacting the eluate with hydroxylapatite under conditions in which  $\alpha$ -glucosidase does not bind to hydroxylapatite and then collecting the unbound fraction enriched in  $\alpha$ -glucosidase.

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The invention therefore provides a method of purifying acid human  $\alpha$ -glucosidase entailing applying a sample containing the  $\alpha$ -glucosidase to two columns. The first column may be either an anion exchange column or an affinity column. Acid  $\alpha$ -glucosidase is applied to the column under binding conditions, so that it becomes bound to the column and it is then eluted. Eluate enriched in acid  $\alpha$ -glucosidase may then be applied either to a hydrophobic interaction column under conditions in which  $\alpha$ -glucosidase binds to the column; or contacted with hydroxylapatite under conditions where  $\alpha$ -glucosidase does not bind. A further eluate when taken from the hydrophobic interaction column is further enriched in  $\alpha$ -glucosidase. The unbound fraction when taken from the hydroxylapatite medium is enriched in  $\alpha$ -glucosidase. The methods are particularly suitable for purifying human acid  $\alpha$ -glucosidase from complex mixtures like the milk of transgenic mammals, such as cows or rabbits for example.

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A preferred material for the first column is Q-Sepharose. Human  $\alpha$ -glucosidase can be bound to such material in low salt buffer and eluted from the column in an elution buffer of higher salt concentration.

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Alternatively, the anion exchange column may be copper chelating Sepharose, phenyl boronate or amino phenyl boronate.

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In another preferred method the affinity column of (a) and (b) is lentil Sepharose.

Regarding step (c) of the method of the invention, when a hydrophobic interaction column is used it is preferably phenyl Sepharose, more preferably Source Phenyl 15. The eluate may be applied to the hydrophobic interaction column in a loading buffer of about 0.5 M or higher molarity ammonium sulphate and eluted from the column with a low salt elution buffer.

Optionally, one or both of the column steps can be repeated as often as desired. The purification method routinely achieves a purity of at least 95%, preferably greater than 99% more preferably greater than 99.9% w/w pure. The methods are also amenable to large-scale production, on initial volumes of at least 100 liters, for example.

A particularly preferred process comprises taking a predominantly whey containing fraction obtained from a transgenic milk, contacting this with hydroxylapatite, either in batch or column format, taking the unbound sample enriched in  $\alpha$ -glucosidase from the hydroxylapatite and then subjecting this to a Q Sepharose chromatography step or steps as hereinbefore defined or as herein described.

A second aspect of the invention provides a method of purifying a heterologous protein from the milk of a transgenic animal comprising:

a) contacting the transgenic milk or a transgenic milk fraction with hydroxylapatite under conditions such that at least a substantial portion of the milk protein species other than the heterologous protein bind to the hydroxylapatite and such that the heterologous protein remains substantially unbound, and;

b) removing the substantially unbound heterologous protein from the hydroxylapatite.

5 The invention therefore also provides for the use of hydroxylapatite in the purification of any heterologous protein from transgenic milk in which the milk proteins can be substantially bound to hydroxylapatite and the heterologous protein is not substantially bound. In this way a rapid single step procedure is possible for separating heterologous protein from substantially all of the other proteins in transgenic milk.

10

The transgenic milk may be contacted directly with the hydroxylapatite without any prior treatment. Preferably though, the transgenic milk is pre-treated, eg by defatting and/or removal of caseins.

15 The heterologous protein is preferably a protein or polypeptide which is not found naturally in the milk of the animal concerned. The heterologous protein may be a non-natural variant of a protein native to the animal and not necessarily a milk protein. The heterologous protein is preferably a protein not normally found in the milk of the animal in question but in a different  
20 animal, preferably, but not necessarily exclusively, found in the milk of that other animal.

The contacting of the milk or milk sample with the hydroxylapatite is carried out for a sufficient time and under suitable conditions of buffer, pH, ionic  
25 strength, other additives, temperature and quantity of hydroxylapatite, such that a substantial portion of the heterologous protein remains free in solution and unbound to the hydroxylapatite. In contrast a substantial portion of the non-heterologous milk proteins are bound to the hydroxylapatite thus advantageously effecting a separation.

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The determination of optimal conditions for ensuring greatest differential in binding of milk proteins and non-binding of a given heterologous protein to hydroxylapatite is something which can readily be performed by one of average skill in the art of protein purification.

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The removal of the substantially unbound heterologous protein preferably involves liquid flow through at least a portion of the hydroxylapatite. The liquid flow may arise as a result of one or more forces selected from pumping, suction, gravity and centrifugal force. The method may advantageously be performed as a batch procedure.

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The hydroxylapatite can be used in the form of a column and therefore optionally the method may be performed as a liquid column chromatography procedure. In a column procedure, the unbound heterologous protein fraction may be collected in the flow-through from the column as part of the column loading process.

15

The quantity of hydroxylapatite used will preferably need to be adjusted in relation to the overall protein content of the milk or milk sample in order to optimize the separation of heterologous protein from the other transgenic milk proteins. This is no more than a matter of routine for the average skilled person in this field.

20

The heterologous protein may be exemplified by any one of the following: lactoferrin, transferrin, lactalbumin, coagulation factors such as factor VIII and factor IX, growth hormone,  $\alpha$ -anti-trypsin, plasma proteins such as serum albumin, C1-esterase inhibitor and fibrinogen, collagen, immunoglobulins, tissue plasminogen activator, interferons, interleukins, peptide hormones, and lysosomal proteins such as  $\alpha$ -glucosidase,  $\alpha$ -L-iduronidase, iduronate-sulfate sulfatase, hexosaminidase A and B, ganglioside activator protein, arylsulfatase A and B, iduronate sulfatase,

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heparan N-sulfatase, galactoceramidase,  $\alpha$ -galactosylceramidase A, sphingomyelinase,  $\alpha$ -fucosidase,  $\alpha$ -mannosidase, aspartylglycosamine amide hydrolase, acid lipase, N-acetyl- $\alpha$ -D-glycosamine-6-sulphate sulfatase,  $\alpha$ - and  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\beta$ -mannosidase, 5 ceramidase, galactocerebrosidase,  $\alpha$ -N-acetylgalactosaminidase, and protective protein and others. The above to include allelic, cognate and induced variants as well as polypeptide fragments of the same.

10 The heterologous protein is preferably one not normally found in the milk of an animal.

In a third aspect the invention provides a method of purifying human acid  $\alpha$ -glucosidase comprising contacting a sample containing human acid  $\alpha$ -glucosidase and contaminating proteins with hydroxylapatite 15 under conditions in which  $\alpha$ -glucosidase does not bind to the hydroxylapatite and then collecting the unbound fraction enriched in  $\alpha$ -glucosidase. This method can be carried out as a batch process for simplicity and the bound and unbound  $\alpha$ -glucosidase separated from the hydroxylapatite by a sedimentation process including centrifugation. 20 Advantageously, hydroxylapatite can provide a one-step purification procedure.

The hydroxylapatite may however be in the form of a column and in which case the unbound fraction may be collected in the flow-through 25 from the column as part of the column loading process.

In accordance with any of the aforementioned aspects of the invention the sample is milk which is preferably produced by a transgenic mammal expressing the  $\alpha$ -glucosidase in its milk. Preferred transgenic milks are 30 those of cow or rabbit for example.

Any of the methods of the invention may further comprise additional steps to eliminate fat and/or caseins from the milk. Thus the methods may further comprise centrifuging the milk and removing fat leaving  
5 skimmed milk. The methods may also further comprise washing removed fat with aqueous solution, recentrifuging, removing fat and pooling supernatant with the skimmed milk. A yet further step may comprise removing caseins from the skimmed milk. When caseins are removed, the methods of the invention preferably comprise a step  
10 selected from the group consisting of high speed centrifugation followed by filtration; filtration using successively decreasing filter sizes; and cross-flow filtration.

The sample preferably has a volume of at least 100 liters.

15 In third aspect the invention provides at least 95%, preferably 99%, more preferably 99.8%, even more preferably at least 99.9% w/w pure human acid  $\alpha$ -glucosidase.

20 The invention provides human acid  $\alpha$ -glucosidase substantially free of other biological materials.

The invention provides human acid  $\alpha$ -glucosidase substantially free of contaminants.

25 The invention provides human acid  $\alpha$ -glucosidase as hereinbefore defined produced by any process of the invention hereinbefore described.

Preferably, the  $\alpha$ -glucosidase of the invention is in a form that is enzymatically active, and taken up at a significant levels in the liver, heart and/or muscle cells of a patient following intravenous injection. Uptake is significant if it results in a statistically significant increase ( $p \leq$   
5 0.05) in enzyme activity in a patient with a deficiency of endogenous enzyme.

The invention further provides a pharmaceutical composition and methods for treating patients deficient in endogenous  $\alpha$ -glucosidase  
10 activity. A suitable pharmaceutical composition for single dose intravenous administration typically comprises at least 0.5 to 20 mg/kg, preferably 2 to 10 mg/kg, most preferably 5 mg/kg of 95%, preferably 99%, more preferably 99.8% even more preferably 99.9% w/w pure human acid  $\alpha$ -glucosidase. Methods of treatment typically entail  
15 intravenously administering a dosage of at least 0.5 to 20 mg/kg, preferably 2 to 10 mg/kg, most preferably 5 mg/kg of 95%, preferably 99%, more preferably 99.8% even more preferably 99.9% w/w pure human acid  $\alpha$ -glucosidase to the patient, whereby the  $\alpha$ -glucosidase is taken up by liver and muscle cells of the patient.

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Thus, the invention provides a pharmaceutical composition for single dosage intravenous administration comprising at least 5 mg/kg of 95%, preferably 99%, more preferably 99.8%, even more preferably 99.9% (w/w) pure human acid  $\alpha$ -glucosidase.

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The invention provides a pharmaceutical composition comprising human acid  $\alpha$ -glucosidase as hereinbefore defined.

The invention provides human acid  $\alpha$ -glucosidase as hereinbefore  
30 defined for use as a pharmaceutical.



The invention provides a method of treating a patient deficient in endogenous  $\alpha$ -glucosidase, comprising administering a dosage of at least 5 mg/kg of 95%, preferably 99%, more preferably 99.8% even more preferably 99.9%, (w/w) pure human acid  $\alpha$ -glucosidase intravenously to the patient, whereby the  $\alpha$ -glucosidase is taken up by liver, heart and/or muscle cells of the patient.

The invention provides for the use of human acid  $\alpha$ -glucosidase as hereinbefore defined for the manufacture of a medicament for treatment of human acid  $\alpha$ -glucosidase deficiency. In twelfth aspect the invention provides for the use of human acid  $\alpha$ -glucosidase as hereinbefore defined for the manufacture of a medicament for intravenous administration for the treatment of human acid  $\alpha$ -glucosidase deficiency.

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#### BRIEF DESCRIPTION OF THE FIGURES

Fig 1. Chromatography profile of rabbit whey on a Q Sepharose FF column. A whey fraction from rabbit (line 60) milk (about 550 ml), prepared by tangential flow filtration (TFF) of the (diluted) skimmed milk, was incubated with solvent/detergent (1% Tween-80, 0.3% TnBP), and loaded on a Q Sepharose FF column (Pharmacia XK-50 column, 18 cm bed height; 250 cm/hr flow rate). The column was washed with (7) column volumes (cv) of buffer A (20 mM sodium phosphate buffer pH 7.0), and the human acid  $\alpha$ -glucosidase fraction was eluted with 3.5 cv buffer A, containing 100 mM sodium chloride. All strongly bound proteins were eluted with about 3 cv 100% buffer B (1 M NaCl, 20 mM sodium phosphate buffer pH 7.0). All column chromatography was controlled by the AKTA system of Pharmacia. Protein was detected on-line by measuring the absorbance at 280 nm. The conductivity was measured online. mAU = milli-Absorbance Units; mS/cm = milli-Siemens/cm.

Fig. 2. Chromatography profile of Q Sepharose FF-purified recombinant human  $\alpha$ -glucosidase fraction on a Phenyl HP Sepharose column.

5 One volume of 1 M ammonium sulphate was added to the Q Sepharose FF human acid  $\alpha$ -glucosidase eluate (obtained with 100 mM sodium chloride, 20 mM sodium phosphate buffer pH 7.0 step; fraction F3 of Fig. 1) while stirring continuously. This sample was loaded on a Phenyl HP Sepharose column (Pharmacia XK-50 column, 14 cm bed height; 150 cm/hr flow rate) at room  
10 temperature (loaded 1 - 1.2 mg  $\alpha$ -glucosidase/ml Sepharose). Before loading, the column was equilibrated in 0.5 M ammonium sulphate, 50 mM sodium phosphate buffer pH 6.0 (= buffer C). After loading the sample, the column was washed with 2 cv of buffer C to remove contaminating proteins like transferrin and serum albumin. Most recombinant human acid  $\alpha$ -  
15 glucosidase was eluted from the Phenyl HP column with 4 cv buffer D (= 50 mM sodium phosphate at pH 6.0 buffer). The strongest bound proteins were eluted first with water, then with 20% ethanol.

Fig. 3. Chromatography profile of a (Phenyl HP Sepharose-purified)  
20 recombinant human  $\alpha$ -glucosidase fraction on Source Phenyl 15 column.

A 2 M ammonium-sulphate, 50 mM sodium phosphate buffer, pH 7.0 was added to the human acid  $\alpha$ -glucosidase eluate from the Phenyl HP column (fraction F4 from Fig. 2), until a final concentration of 0.85 M ammonium  
25 sulphate was reached. The solution was stirred continuously and mildly. The eluate was loaded on a Source Phenyl 15 column (Pharmacia Fineline 100 column, 15 cm bed height; 76 cm/hr flow rate) pre-equilibrated in 0.85 M ammonium sulphate, 50 mM sodium phosphate pH 7.0 buffer (= buffer E). About 2 mg of acid  $\alpha$ -glucosidase can be loaded per ml Source 15 Phenyl in  
30 this column. After loading the sample, recombinant human acid  $\alpha$ -glucosidase was eluted from the (Source 15 Phenyl) column with 10 cv of a

linear gradient from 100% buffer E to 100% buffer F (buffer F = 50 mM sodium phosphate buffer, pH 7.0). Careful pooling of the elution fraction is required (based on purity profiles of the column fractions on SDS-PAGE using Coomassie Brilliant Blue staining) to obtain highly purified  
5 recombinant acid  $\alpha$ -glucosidase. Residual bound proteins were eluted from the column, first with water, and then with 20% ethanol.

Fig. 4. SDS-PAGE analysis of various fractions during the acid  
 $\alpha$ -glucosidase purification procedure. Various fractions obtained during a  
10 recombinant human acid  $\alpha$ -glucosidase purification from rabbit milk (line 60) were diluted in non-reduced SDS sample buffer. The samples were boiled for 5 minutes and loaded on a SDS-PAGE gradient gel (4-12%, Novex).

Proteins were stained with Coomassie Brilliant Blue. Lane 1: Full rabbit milk  
15 ( 40  $\mu$ g); 2. Whey after TFF of skimmed milk ( 40  $\mu$ g); 3. Acid  $\alpha$ -glucosidase eluate fraction from the Q Sepharose FF column ( 30  $\mu$ g); 4. Acid  $\alpha$ -glucosidase eluate fraction from the Phenyl HP column ( 5  $\mu$ g); 5. Acid  $\alpha$ -glucosidase eluate fraction from the Source 15 Phenyl column ( 5  $\mu$ g).

20 The letters refer to protein bands which were identified as: a. rabbit immunoglobulins; b. unknown protein; c. recombinant human acid  $\alpha$ -glucosidase precursor (doublet under these SDS-PAGE conditions); d. rabbit transferrin; e. rabbit serum albumin; f. rabbit caseins; g. rabbit Whey Acidic Protein (WAP), possibly a dimer; h. rabbit Whey Acidic Protein (WAP),  
25 monomer; i. unknown protein, possibly a rabbit WAP variant, or  $\alpha$ -lactalbumin; j. dimer or recombinant human acid  $\alpha$ -glucosidase precursor (doublet under these SDS-PAGE conditions); k. unknown protein (rabbit transferrin, or processed recombinant human acid  $\alpha$ -glucosidase).

30 Fig. 5. HPLC size exclusion profile of purified recombinant human acid  $\alpha$ -glucosidase precursor.

Recombinant human acid  $\alpha$ -glucosidase precursor was purified from transgenic rabbit milk by defatting milk, TFF of skimmed milk, Q FF chromatography, Phenyl HP chromatography. Source 15 Phenyl chromatography, and final filtration. The sample was prepared for the HP-SEC chromatography run as described in Example 1.

Fig. 6. Binding of  $^{125}\text{I}$  human acid  $\alpha$ -glucosidase precursor to various metal-chelating and lectin Sepharoses. Purified human acid  $\alpha$ -glucosidase precursor from rabbit line 60 was radio-labeled with  $^{125}\text{I}$  as described in Example 1. Binding of the labeled enzyme to the metal-chelating Sepharoses ( $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ , glycine, and control) and to the lectin Sepharoses (Concanavalin A and lentil) was done as described in Example 1. Two washing procedures were tested: either a wash with PBS, 0.002% Tween-20 buffer, or a wash with PBS, 0.1% Tween-20, 0.5 M sodium chloride buffer. The binding percentages relate to the total amount of radio-label added to the tubes.

Fig. 7. Chromatographic elution profiles of acid  $\alpha$ -glucosidase-containing fractions on various HIC columns.

Purified acid  $\alpha$ -glucosidase 110 kDa precursor or mature 76 kDa acid  $\alpha$ -glucosidase (A and B; both 5 $\mu\text{g}$ ; recombinant from transgenic mouse milk line 2585) were analyzed on a 1 ml Butyl 4 Fast Flow Sepharose or Octyl 4 Fast Flow Sepharose HiTrap column (Pharmacia, Sweden). A transgenic (line 60; -0-) and non-transgenic (--) whey fraction (prepared by 20,000 g, 60 min centrifugation) were also analyzed on a butyl column (both 200  $\mu\text{l}$ , 25-fold diluted; C). Also a Q Fast Flow fraction (eluted at 100  $\mu\text{l}$  salt from the column; see Fig. 1) of transgenic (line 60; -0-) and non-transgenic (-) whey were loaded on an ether column (both 200  $\mu\text{l}$ , 25-fold diluted; Toyopearl

Ether 650 M (TosoHaas) in a 2.5 ml, 5 cm bed height column; C). The results indicate a strong binding of acid  $\alpha$ -glucosidase to the HIC columns (A and B). Most whey proteins do not bind (C). A nearly pure acid  $\alpha$ -glucosidase was obtained after loading a Q Fast Flow eluate on an ether column (D), where most of the contaminating proteins like serum albumin and transferrin do not bind (SDS-PAGE gels not shown).

The binding buffer in A, B, and C was M ammonium sulphate, 50 mM sodium phosphate pH 7.0. The binding buffer in D was 1.5 M ammonium sulphate, 50 mM sodium phosphate pH 7.0. The flow rate was 1 ml/min. Bound protein was eluted with a linear salt gradient to 50 mM sodium phosphate pH 7.0 in 30 min. All column chromatography was controlled by the AKTA system of Pharmacia. Protein was detected on-line by measuring the absorbance at 280 nm (0.2 cm flow cell). The conductivity was measured on line. mAU= milli-Absorbance units, mS/cm= milli-Siemens/cm.

Fig. 8 Chromatography profiles of transgenic and non-transgenic whey fractions on a Hydroxylapatite column.

Transgenic (----) and non-transgenic (-.-.-.-) rabbit whey, obtained after skimming (by centrifugation) and casein removal (by TFF), were loaded on a Amberchrome column (4.6x150 mm) containing Macro-Prep ceramic hydroxylapatite type I (40  $\mu$ m beads; BioRad) connected to a FPLC system of Pharmacia. Whey fractions obtained after TFF were diluted 5-fold in buffer A (10 mM NaPi pH 6.8), and 0.2 ml was loaded on the column pre-equilibrated in buffer A. The flow rate was 2 ml/min. After loading, bound protein was eluted with a gradient to 500 mM NaPi pH 6.8 in 10 column volumes. Protein was detected by measuring the absorbance at 280 nm (flow cell is 2 mm).

Fig 9. SDS-PAGE analysis of whey fractions from the hydroxylapatite column. Transgenic and non-transgenic rabbit whey were loaded on the Macro-Prep ceramic hydroxylapatite type I column as described in Fig. 8. Flow through and eluate fractions were obtained, which were analyzed on SDS-PAGE (for details of the gels see Fig. 4). A. silver stained SDS-PAGE of transgenic whey run on hydroxylapatite; B. silver stained SDS-PAGE of non-transgenic whey. Up to 6  $\mu$ g protein was loaded.

Figures 10 to 15 are chromatograms of hydroxylapatite chromatography separations of transgenic whey samples in which the samples were loaded on to the column at sodium phosphate buffer ( $\text{NaP}_i$ ) concentrations of 5, 10, 20, 30, 40 or 50 mM respectively. The pH of the buffer was 7.0. The chromatograms show the gradient of sodium phosphate eluting buffer to 400mM, the  $A_{280}$  and the pH of the eluate and the fractions collected.

Figures 16 to 19 are chromatograms of hydroxylapatite chromatography separations as in figures 10 to 15 above except that the pH of the sample was varied whilst the  $\text{NaP}_i$  buffer concentration was retained at 5mM. The pH of the samples fractionated were pH 6.0, 7.0 and 7.5 respectively.

Figure 20 is a chromatogram of an industrial (pilot) scale separation of transgenic milk whey on Q Sepharose FF.

Figure 21 is a chromatogram of hydroxylapatite column chromatography of 0.1M eluate from the Q Sepharose FF column.

Figure 22 is a silver stained SDS-PAGE gel of flow through fractions from a series of hydroxylapatite chromatography separations of 0.1M eluates of Q Sepharose FF.

## DEFINITIONS

5 The term "substantially pure" or "isolated" means human acid  $\alpha$ -glucosidase that has been identified and separated and/or recovered from a component of its natural environment. Usually, the object species is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 10 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent by weight of all macromolecular species present in the composition. Most preferably, the object species is purified to 95%, 99% or 99.9% pure or essential homogeneity (contaminant species cannot be 15 detected in the composition by conventional detection methods) wherein the composition consists essentially of derivatives of a single macromolecular species.

20 A low salt buffer means a buffer with a salt concentration less than 100 mM and preferably less than 50 mM. A high salt buffer means a buffer with a salt concentration greater than 300 mM and preferably at least 500 mM.

## DETAILED DESCRIPTION

25 The present invention provides *inter alia* methods of purifying heterologous proteins from the milk of transgenic animals, preferably human acid  $\alpha$ -glucosidase. The methods are amenable for large-scale production, and result in proteins including  $\alpha$ -glucosidase in a form suitable for therapeutic 30 administration. The methods are particularly suitable for isolating human proteins and in particular human acid  $\alpha$ -glucosidase from milk produced by

transgenic animals. In one aspect the invention provides methods entailing two chromatography steps, one an anion-exchange column or affinity chromatography step, the other a hydrophobic interaction column or using hydroxylapatite in batch or column chromatography format. The two different  
5 separations act in a synergistic fashion substantially eliminating contaminating proteins present in a milk composition. For example, an anion exchange column separates human acid  $\alpha$ -glucosidase from acid whey protein but not completely from serum albumin and transferrin. A hydrophobic interaction column effectively separates human acid  $\alpha$ -  
10 glucosidase from serum albumin and transferrin but not from acid whey protein.

A typical purification procedure may involve additional steps before and after the above column purifications. For example, when human acid  $\alpha$ -glucosidase is purified from milk, fat and caseins are removed from milk  
15 before column chromatography. The procedure can also include further steps to eliminate any viruses that may be present.  $\alpha$ -Glucosidase is then separated from whey proteins and other milk proteins by the two column steps noted above. Each or both of these may be performed more than once  
20 until a desired degree of purification has been achieved. After column chromatography  $\alpha$ -glucosidase is optionally concentrated and resuspended in a storage buffer.

In another aspect the invention provides a procedure involving  
25 hydroxylapatite under optimised conditions wherein the heterologous protein is substantially unable to bind to the matrix whereas the contaminating milk proteins are substantially bound. The method provides a quick and reproducible one step clean up giving a substantial purification of the heterologous protein of interest.



### I. Sources of $\alpha$ -glucosidase

As noted, the methods are particularly suited to the purification of human acid  $\alpha$ -glucosidase from the milk of transgenic animals. Production of  $\alpha$ -glucosidase in the milk of transgenic animals is described by WO 97/05771 (incorporated by reference in its entirety for all purposes). Briefly, regulatory sequences from a mammary gland specific gene, such as  $\alpha$ -s1-casein are operably linked to an  $\alpha$ -glucosidase coding sequence. The transgene is then introduced into an embryo, which is allowed to develop into a transgenic mammals. Female transgenic mammals express the transgene in their mammary gland and secrete human acid  $\alpha$ -glucosidase into milk. For mice, levels up to 4 gram per liter and for rabbit, levels up to 7 gram per liter can be obtained.

Transgenic rabbits are of particular interest since they breed fast, so a production herd can be established in a short time frame, and they produce significant quantities of milk (up to 0.5 liter/week) containing about 150 gram of protein per liter. Transgenic cows (DeBoer et al., WO 91/08216) are also of interest since they produce, at low costs, large quantities of milk (about 10,000 liters/year) containing about 35 gram of protein per liter [Swaigood, *Developments in Dairy Chemistry-1*, Ed. Fox, Elsevier Applied Science Publisher, London (1982) Pp. 1-59]. Goats, sheep, pigs, mice and rats are also appropriate hosts for expression of  $\alpha$ -glucosidase in their milk (see, e.g., Rosen, EP 279,582, Simon et al., *Bio/Technology* 6, 179-183 (1988)).

Other sources of human acid  $\alpha$ -glucosidase include cellular expression systems (e.g., bacterial, insect, yeast or mammalian) and natural sources, such as human tissues (e.g, liver from cadavers).

## II. Defatting Milk

Defatting of the rabbit milk can be done using conventional methods e.g. low-speed centrifugation (about 2000g) with a Hettich Rotanta RP, Sorvall  
5 RC-5B, or a continuous flow centrifugation appliance such as an Elecrem that result in a required efficiency of fat removal. Milk can be collected and frozen directly, or can first be defatted and then frozen. Optionally, separated fat can be washed with water or a low salt buffer, and the wash subsequently re-centrifuged to improve the recovery of product to be  
10 purified. Also other methods as used in the bovine dairy industry for fat removal can be applied (e.g. filtration).

## III. Removal of caseins from milk

15 Caseins can be removed from milk by various methods. Some methods employ either acid treatment or heat shock. For example, in one method, skimmed milk is brought to pH 4.7, incubated for about 30 min, followed by e.g. centrifugation. Optionally, a temperature shock can be applied after adjusting to pH 4.7, from e.g. 10°C to about 35°C, again followed by  
20 (low-speed: ~2000 g for a few minutes) centrifugation. Although this method can be employed in the separation of caseins from milk containing human acid  $\alpha$ -glucosidase, it is not preferred because human acid  $\alpha$ -glucosidase is sensitive to both pH and temperature treatment. Human acid  $\alpha$ -glucosidase activity is in general significantly decreased when the pH drops below 4.5, or  
25 when the temperature is raised above 40°C.

Other methods of separating casein from milk use high-speed centrifugation and/or dead-end filtration and/or tangential flow filtration. Centrifugation can be performed on a large scale using a Powerfuge (hundreds of liters of  
30 skimmed milk) to remove caseins. Since the efficiency of casein removal is not 100% but more like 80-90%, the centrifuged whey is further clarified

before subsequent chromatography. Clarification can be done by either dead-end filtration (i.e., use of filters of successively smaller pore size) or cross-flow filtration (i.e. TFF) can be used.

- 5 Tangential flow filtration gives the best results: clear whey is obtained with high acid  $\alpha$ -glucosidase passage over the membrane (>90% recovery of product can be obtained after diafiltration). Tangential flow filtration (also known as cross flow filtration) is a special way of filtration that leads to less clogging of the membrane due to the recirculated flow transverse to the
- 10 membrane. The advantage in a pharmaceutical (industrial) process is that these types of membranes can be reused after cleaning, in contrast to dead-end filters. As well as being used as a source of acid  $\alpha$ -glucosidase for subsequent purification, whey resulting from TFF can be used to produce food products containing whey. Separated caseins can also be used in food
- 15 production.

In the tangential flow filtration mode, several types of membranes have been tested. Various membranes were found to be suitable (meaning a clear filtrate with high acid  $\alpha$ -glucosidase passage): pores varied from about 0.05

20 to 0.3  $\mu\text{m}$  with a preference for a pore size of 0.1 to 0.2  $\mu\text{m}$ . Processing of a Powerfuge whey fraction over a Biomax 1000k membrane (Millipore) yielded a clear whey filtrate with a passage of human acid  $\alpha$ -glucosidase of 60-80%. The recovery can be increased up to >97% after washing the retentate fraction with a buffer (e.g. 20 mM sodium phosphate buffer pH 7.0) in the

25 so-called diafiltration mode.

Cross-flow filtration can be used to separate caseins from milk without a prior high speed centrifugation step. Clear whey is obtained with a passage of human acid  $\alpha$ -glucosidase of 65-35%. With diafiltration (addition of buffer

30 to maintain volume) the recovery can be increased to >90%. After diafiltration the filtrate has to be concentrated. This can be done easily with

ultrafiltration using e.g. a Biomax 30k (Millipore) membrane or any other membrane with a pore-size so small that acid  $\alpha$ -glucosidase does not pass the membrane.

5     IV. Column Chromatography

One preferred method according to the invention employs two column chromatography steps, one an anion exchange or affinity column, the other a hydrophobic interaction column. The steps can be performed in either order.  
10    Either or both of the steps can be repeated to obtain a higher degree of purity.

Anion exchange columns have two components, a matrix and a ligand. The matrix can be, for example, cellulose, dextrans, agarose or polystyrene. The  
15    ligand can be diethylaminoethyl (DEAE), polyethyleneimine (PEI) or a quaternary ammonium functional group. The strength of an anion exchange column refers to the state of ionization of the ligand. Strong anionic exchange columns, such as those having a quaternary ammonium ligand, bear a permanent positive charge over a wide pH range. In weak anion  
20    exchange columns, such as DEAE and PEI, the existence of the positive charge depends on the pH of the column. Strong anion exchange columns such as Q Sepharose FF, or metal-chelating Sepharose (e.g.,  $\text{Cu}^{2+}$ -chelating Sepharose) are preferred. Anion exchange columns are generally loaded with a low-salt buffer at a pH above the pI of  $\alpha$ -glucosidase.  
25    The calculated pI of  $\alpha$ -glucosidase is 5.4 (SWISS-PROT database). The columns are washed several times in the low-salt buffer to elute proteins that do not bind. Proteins that have bound are then eluted using a buffer of increased salt concentration.

30    Q Sepharose FF is a preferred anion exchange column because this material is relatively inexpensive compared with other anion-exchange

columns and has a relatively large bead size suitable for large scale purification. Under specified conditions Q Sepharose FF binds human acid  $\alpha$ -glucosidase and separates  $\alpha$ -glucosidase sufficiently from the strongest binding (milk) proteins. This is essential since some of these strongly binding proteins, for instance rabbit whey acidic protein (WAP), tend to co-elute with  $\alpha$ -glucosidase in the subsequent hydrophobic interaction chromatography (HIC) steps. To obtain good binding of human acid  $\alpha$ -glucosidase to the Q Sepharose FF, the column is pre-equilibrated in low salt (i.e., less than 50 mM, preferably less than 35 mM such as sodium or potassium phosphate buffer or other suitable salts such as Tris. The pH of the buffer should be about 7.0  $\pm$  1.0 to obtain a good binding of human acid  $\alpha$ -glucosidase to the column. A much higher pH is not suitable because human acid  $\alpha$ -glucosidase is inactivated to some extent. A much lower pH weakens binding of  $\alpha$ -glucosidase to the anion-exchange material.

Human acid  $\alpha$ -glucosidase is then eluted by step-wise or gradient elution at increased salt concentration. Step-wise elution is more amenable to large-scale purification. About 85% of loaded human acid  $\alpha$ -glucosidase can be eluted from a Q FF column in one step (at about 0.1 M salt) with relatively high purity. The main protein contaminants when  $\alpha$ -glucosidase is purified from rabbit milk are rabbit milk-derived proteins like transferrin and serum albumin. Strongly binding milk proteins, such as WAP, elute from Q Sepharose FF with higher salt concentrations, e.g. about 1 M salt.

Optionally, the anion-exchange step can be replaced with an affinity chromatography step, although such is not preferred. Suitable affinity reagents include lectins and antibodies. Lectins are plant-derived carbohydrate binding proteins that have affinity for glycoproteins. Proteins are typically loaded on lectin columns in a buffer of about 150 mM salt and neutral pH containing about 1 mM  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . Glycoproteins can be eluted from such columns using a buffer containing 0.1-0.5 M concentration

of a simple sugar, such as sucrose. Examples of lectin affinity columns includes lectins coupled to Sepharose (or other matrices) such as lentil-Sepharose (reported to be less toxic compared to Concanavalin A). Also, ligands recognizing vicinal diols can be used, such as (amino) phenyl boronate. Monoclonal or polyclonal antibodies to human acid  $\alpha$ -glucosidase can also be used as affinity reagents. Antibodies are typically linked to cyanogen bromide activated Sepharose. Non-specifically bound or weakly bound proteins can be eluted from such a column using a neutral buffer at moderately high salt concentration (i.e., greater than about 0.5 M). Specifically bound  $\alpha$ -glucosidase is eluted using low pH buffer (e.g., 50 mM citrate, pH 3.0). Following elution,  $\alpha$ -glucosidase should be neutralized. Antibody-based affinity purification is not preferred relative to anion exchange, because antibodies are relatively expensive reagents, and as a biologic are subject to FDA review if the ultimate goal of purification is to produce a protein for therapeutic use.

The second column used for isolating human acid  $\alpha$ -glucosidase is a hydrophobic interaction chromatography (HIC) column. HIC columns have two components, a matrix and a ligand. Suitable matrices include Sepharose and polystyrene. Suitable ligands include phenyl-, butyl-, octyl-, and ether- groups. Phenyl-Sepharose<sup>TM</sup> or (Source Phenyl 15 (phenyl group linked to polystyrene column)) are particularly suitable. The loading buffer for HIC chromatography contains a high concentration of a salt that favours hydrophobic interactions. Suitable anions are phosphate, sulphate and acetate. Suitable cations are ammonium, rubidium and potassium. For example, a solution of about 0.5 +/- 0.2 M ammonium sulphate, pH 6 is suitable. Under these conditions, human acid  $\alpha$ -glucosidase binds to the column whereas most other proteins do not.  $\alpha$ -glucosidase can then be eluted with a low salt elution buffer. For example, buffer of 25-100 mM, preferably 50 mM sodium phosphate buffer, pH about 6.0 (+/- 1.0) is suitable).

The relative order of elution of human acid  $\alpha$ -glucosidase and other milk proteins depends on the nature of the column. For example, on a phenyl-Sepharose column,  $\alpha$ -glucosidase binds better than serum albumin. On a (Source Phenyl 15) column the reverse is the case. Transferrin binds more weakly to a Source Phenyl 15 column and a phenyl-Sepharose column. Transferrin binding can be blocked at (e.g. 0.5 M ammonium sulfate).

#### V. Viral Elimination

10

For the removal of viruses, a solvent/detergent step can be incorporated at any point in the procedure, usually after removal of fat and caseins from milk. A specific combination of solvent and detergent, like 0.3% tri-n-butylphosphate (TnBP) combined with 1% Tween-80, is very effective in the removal of enveloped viruses (Horowitz et al (1985) *Transfusion* 25, pp. 516-522). A whey fraction obtained after cross-flow filtration was incubated for 6 hours at 25°C with 0.3% TnBP and 1% Tween-80. After this incubation, the whey was directly loaded on a Q FF chromatography column. After washing the column with the binding buffer, and elution of bound acid  $\alpha$ -glucosidase with salt buffer (see examples), most solvents and detergent were removed from the  $\alpha$ -glucosidase in our product. To ensure removal of residual non-enveloped viruses (such as the small parvo virus), an extra nanofiltration step can be incorporated in one of the last steps of the process. These filters are well defined nowadays (e.g. Planova 15 or 35).

25

#### VI. Resuspension, Storage and Concentration

After recovery from the chromatography column, human acid  $\alpha$ -glucosidase is optionally concentrated and resuspended in a suitable buffer for storage or use. Optionally, human acid  $\alpha$ -glucosidase can be freeze-dried for storage purposes.

30

### Uses of Purified Human Acid $\alpha$ -Glucosidase

Purified human acid  $\alpha$ -glucosidase produced according to the invention finds  
5 use in enzyme replacement therapeutic procedures. A patient having a  
genetic or other deficiency resulting in an insufficiency of enzyme can be  
treated by administering exogenous enzyme to the patient. Patients in need  
of such treatment can be identified from symptoms (e.g., cardiomegaly,  
10 hépatosplenomegaly, increased numbers of lysosomes and markers thereof,  
joint stiffness). Alternatively, or additionally, patients can be diagnosed from  
biochemical analysis of a tissue sample to reveal excessive accumulation of  
a metabolite processed by  $\alpha$ -glucosidase or by enzyme assay using an  
artificial or natural substrate to reveal deficiency of acid  $\alpha$ -glucosidase.  
15 Diagnosis can be made by measuring the particular enzyme deficiency or by  
DNA analysis before occurrence of symptoms or excessive accumulation of  
metabolites (Scriver et al., *supra*, chapters on lysosomal storage disorders).  
 $\alpha$ -Glucosidase storage diseases are hereditary. Thus, in offspring from  
families known to have members suffering from  $\alpha$ -glucosidase, it is  
sometimes advisable to commence prophylactic treatment even before a  
20 definitive diagnosis can be made.

In some methods, human acid  $\alpha$ -glucosidase is administered in purified form  
together with a pharmaceutical carrier as a pharmaceutical composition.  
The preferred form depends on the intended mode of administration and  
25 therapeutic application. The pharmaceutical carrier can be any compatible,  
nontoxic substance suitable to deliver the polypeptides to the patient. Sterile  
water, alcohol, fats, waxes, and inert solids can be used as the carrier.  
Pharmaceutically-acceptable adjuvants, buffering agents, dispersing agents,  
and the like, may also be incorporated into the pharmaceutical compositions.



The concentration of the enzyme in the pharmaceutical composition can vary widely, i.e., from less than about 0.1% by weight, usually being at least about 1% by weight to as much as 20% by weight or more.

5 For oral administration, the active ingredient can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. Active component(s) can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as glucose, lactose, sucrose, mannitol, starch,  
10 cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that may be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide,  
15 edible white ink and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the  
20 atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain colouring and flavouring to increase patient acceptance.

A typical composition for intravenous infusion could be made up to contain  
25 100 to 500 ml of sterile 0.9% NaCl or 5% glucose optionally supplemented with a 20% albumin solution and 100 to 500 mg of enzyme. A typical pharmaceutical compositions for intramuscular injection would be made up to contain, for example, 1 ml of sterile buffered water and 1 to 10 mg of the purified enzyme of the present invention. Methods for preparing parenterally  
30 administrable compositions are described in more detail in various sources, including, for example, *Remington's Pharmaceutical Science* (15th ed., Mack

Publishing, Easton, PA, 1980) (incorporated by reference in its entirety for all purposes).

5 The pharmaceutical compositions of the present invention are usually administered intravenously. Intradermal, intramuscular or oral administration is also possible in some circumstances. The compositions can be administered for prophylactic treatment of individuals suffering from, or at risk of, a lysosomal enzyme deficiency disease. For therapeutic applications, the pharmaceutical compositions are administered to a patient  
10 suffering from established disease in an amount sufficient to reduce the concentration of accumulated metabolite and/or prevent or arrest further accumulation of metabolite. For individuals at risk of lysosomal enzyme deficiency disease, the pharmaceutical composition are administered prophylactically in an amount sufficient to either prevent or inhibit  
15 accumulation of metabolite. An amount adequate to accomplish this is defined as a "therapeutically-" or "prophylactically-effective dose." Such effective dosages will depend on the severity of the condition and on the general state of the patient's health, but will generally range from about 0.1 to 10 mg of purified enzyme per kilogram of body weight.

20

Human acid  $\alpha$ -glucosidase produced in the milk of transgenic animals has a number of other uses. For example,  $\alpha$ -glucosidase, in common with other  $\alpha$ -amylases, is an important tool in production of starch, beer and pharmaceuticals. See Vihinen & Mantsala, *Crit. Rev. Biochem. Mol. Biol.* 24,  
25 329-401 (1989) (incorporated by reference in its entirety for all purpose). Human acid  $\alpha$ -glucosidase is also useful for producing laboratory chemicals or food products. For example, acid  $\alpha$ -glucosidase degrades 1,4 and 1,6  $\alpha$ -glucosidic bonds and can be used for the degradation of various carbohydrates containing these bonds, such as maltose, isomaltose, starch  
30 and glycogen, to yield glucose. Acid  $\alpha$ -glucosidase is also useful for administration to patients with an intestinal maltase or isomaltase deficiency.

Symptoms otherwise resulting from the presence of undigested maltose are avoided. In such applications, the enzyme can be administered without prior fractionation from milk, as a food product derived from such milk (e.g., ice cream or cheese) or as a pharmaceutical composition. Purified recombinant lysosomal enzymes are also useful for inclusion as controls in diagnostic kits for assay of unknown quantities of such enzymes in tissue samples.

### Examples

#### 10     1. Materials and methods:

##### Acid $\alpha$ -glucosidase assay

A 96-well microtiter plate (NUNC) was put on ice, and 20  $\mu$ l 4-MU substrate (4-methyl umbelliferyl- $\alpha$ -D-glucopyranoside; Mellford Labs, London; 2.2 mM in 0.2 M Na Acetate buffer pH 4.3) was added in a well. Sample to be tested (10  $\mu$ l, diluted in PBS (phosphate buffered saline) + 0.5% BSA (w/v; Sigma fraction V)), was added and incubated for 30 min at 37°C. The reaction was stopped with 200  $\mu$ l 0.5 M Na-carbonate buffer (pH 10.5). The microtiter plate was assayed in a fluorometer (excitation wavelength=360 nm; emission wavelength=460 nm). As a standard recombinant human mature acid  $\alpha$ -glucosidase was included in each assay.

##### Radio-iodination of acid $\alpha$ -glucosidase

Recombinant human precursor acid  $\alpha$ -glucosidase purified from transgenic rabbit milk (line 60) was radio-iodinated with the Chloramin T method. Labeling was essentially done as follows: to 0.2 ml of precursor (~0.1 mg) 10  $\mu$ l of Na<sup>125</sup>I (~1 mCi) was added. Chloramin T (50  $\mu$ l; 0.4 mg/ml in PBS) was added, and incubated for 60 seconds. Then, 50  $\mu$ l Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (1 mg/ml in

PBS) and 100  $\mu$ l of a 0.2 mg/ml NaI solution in PBS was added. Free  $^{125}$ I was separated on a PD 10 gel filtration column (Pharmacia) equilibrated in PBS, 0.1% Tween-20, 1 M NaCl, 0.05% sodium azide. Labeled protein was pooled and kept at  $-80^{\circ}\text{C}$ .

5

#### Radio-assay with metal-chelating Sepharoses and lectin Sepharoses

The binding of radio-iodinated precursor acid  $\alpha$ -glucosidase to metal-chelating sepharose was measured to determine whether a specific  
10 metal interacts with the (radio-labeled) enzyme. Also the binding to lectin Sepharoses was determined.

Chelating Sepharose (Pharmacia) was incubated with various salts according to the recommendations of the manufacturer. Essentially the  
15 Sepharoses were prepared as follows: 3.5 ml packed Sepharose beads were diluted in 500 ml water, centrifuged (3500 rpm, 10 minutes), and after removal of the supernatant, the beads were resuspended in either 50 ml  $\text{CuCl}_2$  (257 mg),  $\text{ZnCl}_2$  (215 mg), ferric-sulphate (400 mg), or ferrous-sulphate (417 mg). After overnight incubation (rotating), the beads  
20 were washed 3 times with water, and then washed with PBS, 0.1% Tween-20, 1 M NaCl, and stored in 50 ml water at  $4^{\circ}\text{C}$ . For the binding experiment, 0.5 ml of the Sepharose beads were washed 5 times with PBS, 0.02% Tween-20, or PBS, 0.1% Tween-20, 0.5 M NaCl. Radio-labeled precursor enzyme (50  $\mu$ l in PBS, 0.1% Tween-20;  $\sim 50,000$  cpm) was added  
25 to 0.5 ml beads suspension, and incubated (rotating) overnight at room temperature. Sepharose beads were washed 4 times with PBS, 0.02% Tween-20, and the amount of bound label was counted in a liquid scintillation counter.

30

## 2. Skimming (defatting) of the transgenic milk

5 A. Milk was thawed in water bath at 25°C while shaking. Then the milk was diluted 2-fold in water to maximize the recovery of the target protein and put into centrifugation bottles or tubes.

10 The milk was defatted by centrifugation at 2800 x g, for 15-30 min. at 4°C. The fat was removed with a spoon or by means of suction. Also full (undiluted) milk was centrifuged under the same conditions. The fat fraction obtained was: (1) washed with water and re-centrifuged, or (2) another batch was washed with a low salt buffer, and re-centrifuged. The skimmed milk and the wash fraction (after re-centrifugation) were pooled for further processing.

15 B. Milk was thawed in water bath at 25°C while shaking, then the milk was put into an Elecrem centrifuge, using continuous centrifugation. The fat fraction was recovered, diluted in water, and re-centrifuged to maximize the recovery of human acid  $\alpha$ -glucosidase in the pooled skimmed milk. A  
20 recovery of > 90% can be obtained.

## 3. Preparation of the whey fraction from skimmed transgenic milk using centrifugation and dead-end filtration

25 The removal of caseins from (diluted) skimmed rabbit milk was obtained by continuous centrifugation, at 20,000 x g, for 30-45 min at 5-20°C in a Powerfuge (Carr). The resulting whey fraction was made suitable for chromatography by dead-end filtration.

30 Dead-end filtration: first a CP15 or AP15 prefilter (Millipore) was used, followed by subsequent filtration over 1.2  $\mu$ m RA, 0.8  $\mu$ m AA, 0.65  $\mu$ m DA

and 0.45  $\mu\text{m}$  HA membrane filters (Millipore, disc-filters with a diameter of 47 mm) at a mild under-pressure. When clogging of filters occurred, new filters were used. The filtrate obtained after 0.45  $\mu\text{m}$  membrane filtration was suitable for chromatography. The recovery of the target protein after centrifugation with the Carr Powerfuge was in general about 60-80%. Dead-end filtration resulted in a minimal loss of human acid  $\alpha$ -glucosidase activity, in general < 3%.

#### 4. Preparation of the whey fraction from skimmed transgenic milk using TFF

10

Whey was prepared out of about 4.5 liter of diluted skimmed rabbit milk by TFF. A Biomax 1000 (0.5  $\text{m}^2$ ) membrane cassette was placed in a cassette holder connected to a Proflux MA from Millipore. This membrane was chosen because it gives a very good retention of casein micelles (meaning the filtrate is very clear) and a passage of human acid  $\alpha$ -glucosidase of about 30-60%. The process conditions were as follows:

15

P-inlet = 1.0 bar, P-outlet = 0.7 bar, P-filtrate = 0.7 bar, TransMembrane Pressure (TMP) = 0.15 bar, flux =  $\sim 15 \text{ L/hr/m}^2$ , process temperature = 10-35°C, preferably about 20°C (room temperature). To improve the recovery of human acid  $\alpha$ -glucosidase in the filtrate, the retentate was diluted with a low salt buffer, e.g. 20 mM sodium phosphate buffer at a pH of 7.0. After about 6 diafiltration volumes, the recovery of human acid  $\alpha$ -glucosidase activity in the filtrate was > 80%. Due to the diafiltration, the volume of the whey fraction (= filtrate) had increased dramatically. The filtrate was concentrated about 7 times by ultrafiltration using a Biomax 30 membrane (Millipore; 0.5  $\text{m}^2$ ) in the same TFF device. This type of membrane is impermeable to  $\alpha$ -glucosidase. A flux of 50  $\text{L/hr/m}^2$  can easily be obtained in this step. The TMP was 1.0 bar. No activity was detected in the filtrate, but all activity was recovered in the retentate fraction. If the permeate contains too much sodium chloride, diafiltration was done with 20

20

25

30

mM sodium phosphate pH 7.0 buffer, to decrease the sodium chloride concentration below 5 mM.

5. Virus inactivation by solvent/detergent (S/D)

5

Virus inactivation (at least of enveloped viruses) of the whey fraction was obtained by incubating the whey in the presence of 1% Tween-80 and 0.3% tri-n-butylphosphate (TnBP) while stirring continuously and mildly, for 4-8 hr (preferably 6 hr) at 25°C. No significant loss of  $\alpha$ -glucosidase activity was observed (< 10%).

10

6. Binding of human acid  $\alpha$ -glucosidase present in filtered whey or whey fraction to Q Sepharose Fast Flow

15 Q Sepharose Fast Flow (QFF; Pharmacia) chromatography (Pharmacia XK-50 column, 15 cm bed height; 250 cm/hr flow rate; all column chromatography controlled by the AKTA system of Pharmacia; protein was detected on-line by measuring the absorbance at 280 nm) was done using the following protocol:

20

1. the column was equilibrated in 20 mM sodium phosphate buffer, pH 7.0 (buffer A).

2. the S/D-incubated whey fraction (about 500 to 600 ml) was loaded.

25

3. after loading the whey fraction, the column was washed with 7 column volumes (cv) of buffer A.

30

4. the human acid  $\alpha$ -glucosidase fraction was eluted from the Q FF column with 3.5 cv buffer A, containing 100 mM sodium chloride.

5. all strongly bound proteins were eluted with about 3 cv 100% buffer B, containing 1 M sodium chloride in 20 mM sodium phosphate buffer pH 7.0.

A representative elution profile of a Q FF chromatography run is shown in Fig. 1. In this specific run, the whey sample loaded on the Q FF column was S/D pretreated. Essentially the same elution profiles were obtained in a whey fraction, which was not subjected to S/D treatment, was loaded on the Q FF column. No Tween-80 or TnBP could be detected in the recombinant human acid  $\alpha$ -glucosidase fraction eluting in buffer A containing 100 mM sodium chloride. Essentially all Tween-80 and TnBP could be detected in the (unbound) flow through fraction. The recovery of recombinant human acid  $\alpha$ -glucosidase (Step 4) was about 80-85%. About 15% of the  $\alpha$ -glucosidase activity was present in the fraction eluting with 100% buffer B.

#### 7. Binding of Q FF Sepharose Human Acid $\alpha$ -Glucosidase Containing Fraction to Phenyl-Sepharose High Performance

One volume of 1 M ammonium sulphate was added to the Q FF Sepharose human acid  $\alpha$ -glucosidase eluate containing the major human acid  $\alpha$ -glucosidase fraction (obtained with 0.1 M sodium chloride, 20 mM sodium phosphate buffer pH 7.0; see Example 6) while stirring continuously. Phenyl HP (Pharmacia) column chromatography (Pharmacia XK-50 column, 14 cm bed height; 150 cm/hr flow rate) was done at room temperature using the following protocol:

1. the column was equilibrated in 0.5 M ammonium sulphate, 50 mM sodium phosphate buffer pH 6.0 (buffer C).

2. the 0.5 M ammonium sulphate-incubated human acid  $\alpha$ -glucosidase eluate was loaded. The dynamic capacity was about 1.2 mg human acid  $\alpha$ -glucosidase/ml Phenyl Sepharose High Performance.



3. after loading the sample, the column was washed with 2 cv of buffer C.
4. most human acid  $\alpha$ -glucosidase was eluted from the Phenyl HP column with 4 cv buffer D (50 mM sodium phosphate buffer at pH 6.0).
5. the strongest binding proteins were eluted first with water, and then with 20% ethanol.
- 10 A representative elution profile of a Phenyl Sepharose HP chromatography run is shown in Fig. 2.

The recovery of human acid  $\alpha$ -glucosidase activity in step 4 was generally > 85%.

15

8. Binding and Elution of Human Acid  $\alpha$ -Glucosidase Fraction from the Phenyl HP Column on Source Phenyl 15.

- 20 A 2 M ammonium-sulphate, 50 mM sodium phosphate buffer, pH 7.0 was added to the human acid  $\alpha$ -glucosidase eluate from the Phenyl HP column, until a final concentration of 0.85 M ammonium sulphate was reached. The solution was stirred continuously and mildly.

25 Source Phenyl 15 (Pharmacia) chromatography (Pharmacia Fineline 100 column, 15 cm bed height; 76 cm/hr flow rate) was done using the following protocol:

1. the column was equilibrated in 0.85 M ammonium sulphate, 50 mM sodium phosphate pH 7.0 buffer (buffer E).

30

2. the ammonium sulphate-diluted human acid  $\alpha$ -glucosidase eluate from Phenyl HP was loaded on the column. The dynamic capacity was about 2 mg recombinant human acid  $\alpha$ -glucosidase/ml Source 15 Phenyl.

5 3. after loading the sample, human acid  $\alpha$ -glucosidase was eluted from the Source 15 Phenyl column with 10 cv of a linear gradient from 100% buffer E to 100% buffer F (buffer F: 50 mM sodium phosphate buffer, pH 7.0). Careful pooling of the elution fraction is required (based on purity profiles of the column fractions on SDS-PAGE using Coomassie Brilliant Blue staining)  
10 since some contaminating proteins elute directly after  $\alpha$ -glucosidase.

4. residual bound proteins were eluted from the column with water and/or 20% ethanol.

15 A representative elution profile of a Source 15 Phenyl chromatography run is shown in Fig. 3.

The recovery of human acid  $\alpha$ -glucosidase activity in the pooled fraction (step 4) was generally > 70%.

20

#### 9. Final filtration steps: ultra-, dia-, sterile-, and nano-filtration

The pooled human acid  $\alpha$ -glucosidase fractions from the Source 15 Phenyl column were concentrated by ultrafiltration in a TFF mode over a 0.1 m<sup>2</sup> Biomax 30 membrane connected to the Proflux M12 system of Millipore.  
25 After a 7-fold concentration, the retentate was diafiltered in 10 mM sodium phosphate buffer, pH 7.0 (about 6 diafiltration volumes were used). Finally the acid  $\alpha$ -glucosidase fraction was sterile filtered (0.2  $\mu$ m dead-end filters). The recovery of human acid  $\alpha$ -glucosidase after these filtration steps was >  
30 85%. Optionally a virus removal step can be incorporated: virus removal filters (nanofilters) like Planova 15 and 35 are feasible.

#### 10. SDS-PAGE and HP-SEC analysis of purified human acid $\alpha$ -glucosidase

Purified human acid  $\alpha$ -glucosidase was analyzed by silver-stained  
5 SDS/PAGE and Size-Exclusion HPLC (HP-SEC). Fig. 4 shows a Coomassie  
Brilliant Blue-stained SDS-PAGE gel (4-12%, NuPage) of various milk  
fractions obtained during the purification run. Similar SDS-PAGE gels were  
visualized by silver-staining. A few minor bands were present. Western  
10 blotting of the gels with a polyclonal antibody against acid  $\alpha$ -glucosidase,  
identified most of these minor bands as dimers and processed forms of the  
precursor acid  $\alpha$ -glucosidase. At least 2 host-related impurities were  
present in the purified recombinant human acid  $\alpha$ -glucosidase preparation.  
The amount of these host-related impurities quantitated by densitometric  
15 scanning of the gel was around 1% of total protein loaded. Purified  
recombinant human acid  $\alpha$ -glucosidase was also analyzed on a size  
exclusion column connected to a High Performance Liquid Chromatography  
System (HP-SEC). Results are shown in Fig. 5. The size exclusion column  
is able to separate proteins essentially on the basis of their molecular  
20 weight. Thus in principle this column is able to visualize and quantitate  
protein impurities of different molecular weights compared with the 110 kDa  
precursor  $\alpha$ -glucosidase. As expected, the main protein peak was the  
recombinant human acid  $\alpha$ -glucosidase precursor; peak surface analysis  
indicated that this peak was 99% of the total surface area of all visualized  
peaks. The molecular weight of the 110 kDa  $\alpha$ -glucosidase monomer was  
25 estimated on this column to be 127 kDa. Some other small peaks were  
visible. On the basis of their elution profiles they were thought to have  
molecular weight of about 240 kDa (the 110 kDa  $\alpha$ -glucosidase dimer),  
about 67 kDa (serum albumin), and about 20 kDa (unknown). Also some  
protein was present in the high molecular weight area.

### 11. Purification of human acid $\alpha$ -glucosidase using metal-chelating and lectin Sepharoses

5 Various metal-chelating Sepharoses were prepared according to the recommendation of the manufacturer (Pharmacia). Radio-labeled human precursor acid  $\alpha$ -glucosidase was incubated overnight with the various Sepharoses (for details: see example 1). After removal of the unbound label by washing, radioactivity bound to the beads was measured in a liquid scintillation counter. The results are shown in Fig. 6. Clearly, the

10  $\text{Cu}^{2+}$ -chelating Sepharose is binding the radio-labeled human precursor acid  $\alpha$ -glucosidase very good. Thus this ligand might be suitable for purification of the enzyme from milk and other sources, in contrast to the  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$  Sepharoses.

15 The radio-labeled human precursor acid  $\alpha$ -glucosidase also binds well to lectin Sepharoses like Concanavalin A (as expected), but unexpectedly also to lentil Sepharose (Fig. 5). Thus also lentil Sepharose is likely to be suitable for purification of acid  $\alpha$ -glucosidase from milk.

### 20 12. Purification of human acid $\alpha$ -glucosidase using various HIC media

Purified acid  $\alpha$ -glucosidase and rabbit milk fractions were incubated with other HIC media than the Phenyl Sepharoses. In Fig. 7 the results are shown of chromatography experiments with column containing butyl, octyl,

25 an ether ligands coupled to Sepharose (Pharmacia) and/or Toyopearl (TosoHaas) beads. Under conditions normal for HIC,  $\alpha$ -glucosidase was found to bind more or less tightly to the various media.

13. Purification of human acid  $\alpha$ -glucosidase using Hydroxylapatite-  
Experiment 1

5

Hydroxylapatite was tried for its ability to separate recombinant human acid  $\alpha$ -glucosidase from contaminating (whey) proteins. Hydroxylapatite is a crystalline form of calcium phosphate. Binding of proteins is mediated through the carboxyl and amino groups of the protein and  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  groups of the hydroxylapatite crystal lattice (Current protocols in Protein Science, eds. J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, P.T. Wingfield. John Wiley & Sons Inc. (1995), suppl. 8.6.9-8.6.12). Electrostatic interactions and specific effects are involved in the binding of neutral and acidic proteins to the  $\text{Ca}^{2+}$  sites, although the interaction of many proteins with hydroxylapatite can not be explained by the pI alone. DNA also binds to the matrix due to the charged phosphate backbone (Current protocols in Protein Science, eds. J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, P.T. Wingfield. John Wiley & Sons Inc. (1995), suppl. 8.6.9-8.6.12).

20

Transgenic and non-transgenic rabbit whey were loaded on a column containing ceramic hydroxylapatite type I (BioRad) at low salt concentration. After loading, bound protein was eluted with a gradient to 400 mM sodium phosphate ( $\text{NaP}_i$ ) pH 6.8. The chromatography profiles shown in Figure 8 clearly show an increased flow through of the transgenic whey compared with the non-transgenic whey. SDS-PAGE analysis using silver staining (Figure 9) clearly indicated that this fraction contains recombinant human acid  $\alpha$ -glucosidase, together with WAP protein. Nearly all other whey proteins were bound to the column (the x axis of Figure 8 shows the fraction numbers corresponding to the

30

fraction numbers at the top of the lanes of the gels in Figure 9). Acid  $\alpha$ -glucosidase activity assays indicated that most activity was in the flow through fractions, and less than 5% was bound to the column.

5 These results clearly show that, unexpectedly, the heterologous protein (recombinant human) acid  $\alpha$ -glucosidase does not bind to hydroxylapatite, while nearly all other whey proteins do. This means that the hydroxylapatite column can equally well be used as a step in the purification process; additional to or instead of Source 15Phe, or  
10 even instead of both HIC columns. The hydroxylapatite can of course be used on a polishing step in a more complicated overall procedure. Hydroxylapatite can also be used as a first step (capturing of impurities), followed by e.g. Q Sepharose chromatography or gel filtration. With hydroxylapatite high flow rates can be obtained, the column is easy to  
15 clean (sanitize), and possible leakage of small amounts of calcium phosphate is not hazardous when injected into humans (e.g. bone contains calcium phosphate). The supplier can provide drug master files (DMF).

20 14. Purification of human acid  $\alpha$ -glucosidase using hydroxylapatite-Experiment 2

Transgenic whey from rabbit (containing about 3% (w/w) recombinant human acid  $\alpha$ -glucosidase in 20mM  $\text{NaP}_i$  buffer pH 7.0 was made by tangential flow  
25 filtration (TFF). The transgenic rabbit whey was diluted with either water or sodium phosphate buffer so as to give a series of transgenic whey samples in which the final concentration of sodium phosphate ( $\text{NaP}_i$ ) ( $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ ) buffer was 5, 10, 20, 30, 40 or 50 mM.

30 The column was equilibrated with 5, 10, 20, 30, 40 or 50mM  $\text{NaP}_i$  pH 6.0 to correspond with the buffer strength of the sample to be loaded. Samples of

1 ml (containing 2.5 mg protein) were individually loaded onto 2.5 ml ceramic Hydroxy Apatite (cHT) type 1 (BioRad) columns (bed height 15 cm). The column was washed after loading with 5cv of an equilibration buffer in order to remove any unbound proteins. The bound proteins were then eluted with  
5 a sodium phosphate gradient from the molarity of the sample in question to 400 mM. 1.9ml fractions were taken from the column eluate and then stored at 4°C until SDS-PAGE analysis.

Figure 10 to 15 show the chromatographic traces obtained on  
10 hydroxylapatite chromatography of the whey samples 5, 10, 20, 30, 40 and 50mM NaPi buffer, pH 7.0 respectively. In figures 12, 13 and 14 (samples= 30, 40 and 50mM NaPi, pH 7.0 respectively), the left hand peak on the trace represents at least a portion of the flow through material and the peak areas in these figures are significantly greater than those to be seen in from the  
15 corresponding peaks in Figures 10 and 15 (samples= 5 and 60mM NaPi, pH 7.0 respectively).

Silver stained SDS-PAGE analysis of fractions showed that at 5, 10 and 20 mM sodium phosphate the majority of the  $\alpha$ -glucosidase was to be found in  
20 the flow through, whereas substantially all of the whey proteins were bound to the cHT beads. At 30, 40 and 50 mM NaPi the majority of the  $\alpha$ -glucosidase remain in the flow through but the amount of whey protein in the flow through was increased.

25 The experiment shows how a good purification with acceptable recovery of protein can be achieved for  $\alpha$ -glucosidase from transgenic whey samples at a sodium phosphate buffer sample concentration of between 5 and 20mM. Where a greater purification with lesser recovery is required then a lower sample buffer concentration may be used.

30

15. Purification of human acid  $\alpha$ -glucosidase using hydroxylapatite-  
Experiment 3

As noted in Experiment 2 above, transgenic rabbit whey containing about  
5 3% (w/w) recombinant human acid  $\alpha$ -glucosidase made by tangential flow  
filtration (TFF). The transgenic whey was diluted with water to give a final  
concentration of sodium phosphate ( $\text{NaP}_i$ ) buffer of 5mM at pH 7.2. 500  $\mu\text{l}$  of  
the diluted whey containing about 2.5mg protein was loaded on 2.5 ml  
ceramic Hydroxy Apatite (cHT) type 1 (BioRad) columns (bed height 15 cm).  
10 Each column was equilibrated with 5 mM sodium phosphate buffer at pH 6.0,  
6.5, 7.0 or 7.5. (Figures 16 to 19). After sample loading the column was  
washed with 5cv of equilibration buffer. The bound proteins were eluted at a  
flow rate of 723cm/hr with a gradient to 400 mM sodium phosphate buffer at  
pH 6.0, 6.5, 7.0 or 7.5 respectively. 1.0ml fractions were analyzed for  
15 protein content by SDS-PAGE stained with silver.

Looking at the results of SDS-PAGE gels of the fractions stained with silver,  
one can see that at pH 6.0  $\alpha$ -glucosidase is only bound weakly to the cHT  
(ie there was some flow through), while substantially all whey proteins were  
20 bound more strongly to the cHT. At pH 6.5, about 90% of the  $\alpha$ -glucosidase  
was in the flow through of the column and a low molecular weight (LMW)  
protein, probably whey acidic protein (WAP), was also in the flow through  
with the  $\alpha$ -glucosidase but was somewhat retarded on the column. At pH  
7.0, all of the  $\alpha$ -glucosidase as well as most of the LMW protein (probably  
25 WAP) and the HMW proteins (probably Immunoglobulins) were in the flow  
through; an about 80 kD protein (probably transferrin) was also in the flow  
through but was somewhat retarded on the column.

Base on these results pH 6.5 would seem optimal for separation of  $\alpha$ -  
30 glucosidase from whey proteins.



16. Purification of human acid  $\alpha$ -glucosidase using Q Sepharose FF and hydroxylapatite

5 Transgenic whey (containing recombinant human acid  $\alpha$ -glucosidase) made by tangential flow filtration was processed in a pilot plant facility by applying it to Q Sepharose FF (25 liter column volume) (Amersham Pharmacia Biotech) in 20 mM sodium phosphate ( $\text{NaP}_i$ ) pH 7.0 buffer. (Figure 20). The column was equilibrated with 4cv 50mM  $\text{NaP}_i$ , pH 7.0 and then 2cv 20mM  $\text{NaP}_i$ , pH 7.0. The  $\alpha$ -glucosidase containing fraction was eluted with 2.7cv  
10 0.1M NaCl pH 7.0. A 47.3 liter sample was taken and this contained 265g protein. A sample of the 0.1M fraction was dialysed (3,500 Dalton molecular weight cut off, Spectra Por) against 10 mM sodium phosphate ( $\text{NaP}_i$ ) pH 6.5 buffer.

15 60 ml of the dialysed 0.1M sample (3.91 mg/ml protein, 1.33 mS/cm) was applied to a 30 ml cHT type 1 column (XK 16/15) (BioRad) at a flowrate of 150cm/hr (5ml/min). (Figure 21). The column was washed after sample loading with 5cv of equilibration buffer (10mM  $\text{NaP}_i$ , pH 6.5) 10ml fractions were collected and the  $\alpha$ -glucosidase was found in the flow through,  
20 whereas the majority of the whey proteins bound to the cHT beads. The bound proteins were eluted with a linear gradient of 10 to 400 mM sodium phosphate buffer. This step decreased the impurity levels in the  $\alpha$ -glucosidase containing Q Sepharose FF fraction from 90% to <0.5% in the flow through fraction of the cHT column. The recovery of  $\alpha$ -glucosidase was  
25 greater than 80%. Figure 21 shows the chromatogram of the sample run on the cHT column.

Figure 22 shows a silver stained SDS-PAGE gel showing the flow through fractions from cHT columns (lanes 1-3, 5-7 and 9-11); molecular weight  
30 standards (lane 4) and sample of QFF eluate loaded onto the cHT column (lane 12).

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

10

**Claims:**

1. A method of purifying human acid  $\alpha$ -glucosidase comprising:

5 (a) applying a sample containing human acid  $\alpha$ -glucosidase and contaminating proteins to an anion exchange or affinity column under conditions in which the  $\alpha$ -glucosidase binds to the column;

10 (b) collecting an eluate enriched in  $\alpha$ -glucosidase from the anion exchange or affinity column;

(c) applying the eluate to

15 (i) a hydrophobic interaction column under conditions in which  $\alpha$ -glucosidase binds to the column and then collecting a further eluate further enriched in  $\alpha$ -glucosidase, or

20 (ii) contacting the eluate with hydroxylapatite under conditions in which  $\alpha$ -glucosidase does not bind to hydroxylapatite and then collecting the unbound fraction enriched in  $\alpha$ -glucosidase.

2. The method of claim 1, wherein the column in steps (a) and (b) is an anion exchange column.

25

3. The method of claim 1 or claim 2, wherein the anion exchange column is Q-Sepharose.

4. The method of claim 3, wherein the sample is applied to the Q-Sepharose column in low salt buffer and is eluted from the column in an elution buffer of higher salt concentration.
- 5 5. The method of claim 1 or claim 2, wherein the anion exchange column is copper chelating Sepharose.
6. The method of Claim 1, wherein the affinity column is lentil Sepharose.
- 10 7. The method of claim 1 or claim 2, wherein the hydrophobic interaction column is phenyl Sepharose.
8. The method of claim 1 or claim 2, wherein the hydrophobic  
15 interaction column is Source Phenyl 15.
9. The method of claim 8, wherein the eluate is applied to the hydrophobic interaction column in a loading buffer of about 0.5 M ammonium sulphate and is eluted from the column with a low salt  
20 elution buffer.
10. The method of any one of claims 1 to 9, further comprising repeating steps (a) and (b) and/or (c) until the  $\alpha$ -glucosidase has been purified to 95%, preferably 99%, more preferably 99.9% w/w pure.
- 25 11. The method of any one of claims 1 to 10, wherein the sample is milk produced by a transgenic mammal expressing the  $\alpha$ -glucosidase in its milk.
- 30 12. The method of claim 11, wherein the transgenic mammal is a cow.

13. The method of claim 11, wherein the transgenic mammal is a rabbit.
- 5 14. The method of any one of claims 11 to 13, further comprising centrifuging the milk and removing fat leaving skimmed milk.
15. The method of claim 14, further comprising washing removed fat with aqueous solution, recentrifuging, removing fat and pooling  
10 supernatant with the skimmed milk.
16. The method of 15, further comprising removing caseins from the skimmed milk.
- 15 17. The method of claim 16, wherein the removing of caseins comprises a step selected from the group consisting of:  
high speed centrifugation followed by filtration;  
filtration using successively decreasing filter sizes; and  
cross-flow filtration.
- 20 18. The method of any preceding claim, wherein the sample has a volume of at least 100 liters.
19. At least 95%, preferably at least 99%, more preferably at least  
25 99.9% w/w pure human acid  $\alpha$ -glucosidase.
20. Human acid  $\alpha$ -glucosidase substantially free of other biological materials.
- 30 21. Human acid  $\alpha$ -glucosidase substantially free of contaminants.

22. Human acid  $\alpha$ -glucosidase of any one of claims 19 to 21 produced by the process of any one of claims 1 to 18.

5 23. A pharmaceutical composition for single dosage intravenous administration comprising at least 5mg/kg of at least 95%, preferably at least 99%, more preferably at least 99.9% (w/w) pure human acid  $\alpha$ -glucosidase.

10 24. A pharmaceutical composition comprising human acid  $\alpha$ -glucosidase as claimed in any one of claims 19 to 22.

25. Human acid  $\alpha$ -glucosidase of any one of claims 19 to 22 for use as a pharmaceutical.

15 26. A method of treating a patient deficient in endogenous  $\alpha$ -glucosidase, comprising administering a dosage of at least 5mg/kg of at least 95%, preferably at least 99%, more preferably at least 99.9% (w/w) pure human acid  $\alpha$ -glucosidase intravenously to the patient, whereby the  $\alpha$ -glucosidase is taken up by liver, heart and/or muscle cells  
20 of the patient.

27. The use of human acid  $\alpha$ -glucosidase of any one of claims 19 to 22 for the manufacture of a medicament for treatment of human acid  $\alpha$ -glucosidase deficiency.

25

28. The use of human acid  $\alpha$ -glucosidase of any one of claims 19 to 23 for the manufacture of a medicament for intravenous administration for the treatment of human acid  $\alpha$ -glucosidase deficiency.

30

29. A method of purifying an heterologous protein from the milk of a transgenic animal comprising:

- 5 a) contacting the transgenic milk or a transgenic milk fraction with a hydroxylapatite under conditions such that at least a substantial number of the milk protein species other than the heterologous protein bind to the hydroxylapatite and the heterologous protein remains substantially unbound, and;
- 10 b) removing the substantially unbound heterologous protein.

30. A method as claimed in claim 29, wherein the removal of the substantially unbound heterologous protein involves liquid flow through at least a portion of the hydroxylapatite.

15

31. A method as claimed in claim 30, wherein the liquid flow arises due to one or more forces selected from pumping, suction, gravity and centrifugal force.

20

32. A method as claimed in any of claims 29 to 31 being a batch procedure.

25

33. A method as claimed in any of claims 29 to 32, wherein the hydroxylapatite is in the form of a column, optionally the method is a liquid column chromatography procedure.

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34. A method as claimed in any of claims 29 to 33, wherein the heterologous protein is selected from lactoferrin, transferrin, lactalbumin, factor IX, growth hormone,  $\alpha$ -anti-trypsin, lactoferrin, transferrin, lactalbumin, coagulation factors such as factor VIII and factor IX, growth hormone,  $\alpha$ -anti-trypsin, plasma proteins such as serum albumin, C1-esterase inhibitor and

fibrinogen, collagen, immunoglobulins, tissue plasminogen activator, interferons, interleukins, peptide hormones, and lysosomal proteins such as  $\alpha$ -glucosidase,  $\alpha$ -L-iduronidase, iduronate-sulfate sulfatase, hexosaminidase A and B, ganglioside activator protein, arylsulfatase A and B, iduronate sulfatase, heparan N-sulfatase, galactoceramidase,  $\alpha$ -galactosylceramidase A, sphingomyelinase,  $\alpha$ -fucosidase,  $\alpha$ -mannosidase, aspartylglycosamine amide hydrolase, acid lipase, N-acetyl- $\alpha$ -D-glycosamine-6-sulphate sulfatase,  $\alpha$ - and  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\beta$ -mannosidase, ceramidase, galactocerebrosidase,  $\alpha$ -N-acetylgalactosaminidase, and protective protein and others including allelic, cognate or induced variants as well as polypeptide fragments of the same.

35. A method as claimed in any of claims 29 to 34, wherein the heterologous protein is not one normally found in the milk of an animal.

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36. A method of purifying human acid  $\alpha$ -glucosidase comprising contacting a sample containing human acid  $\alpha$ -glucosidase and contaminating proteins with hydroxylapatite under conditions in which  $\alpha$ -glucosidase does not bind to the hydroxylapatite and then collecting the unbound fraction enriched in  $\alpha$ -glucosidase.

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37. The method of claim 3, wherein the hydroxylapatite is in the form of a column and the unbound fraction is collected in the flow-through.

38. A method of purifying human acid  $\alpha$ -glucosidase substantially as hereinbefore described and with reference to the examples and accompanying drawings.

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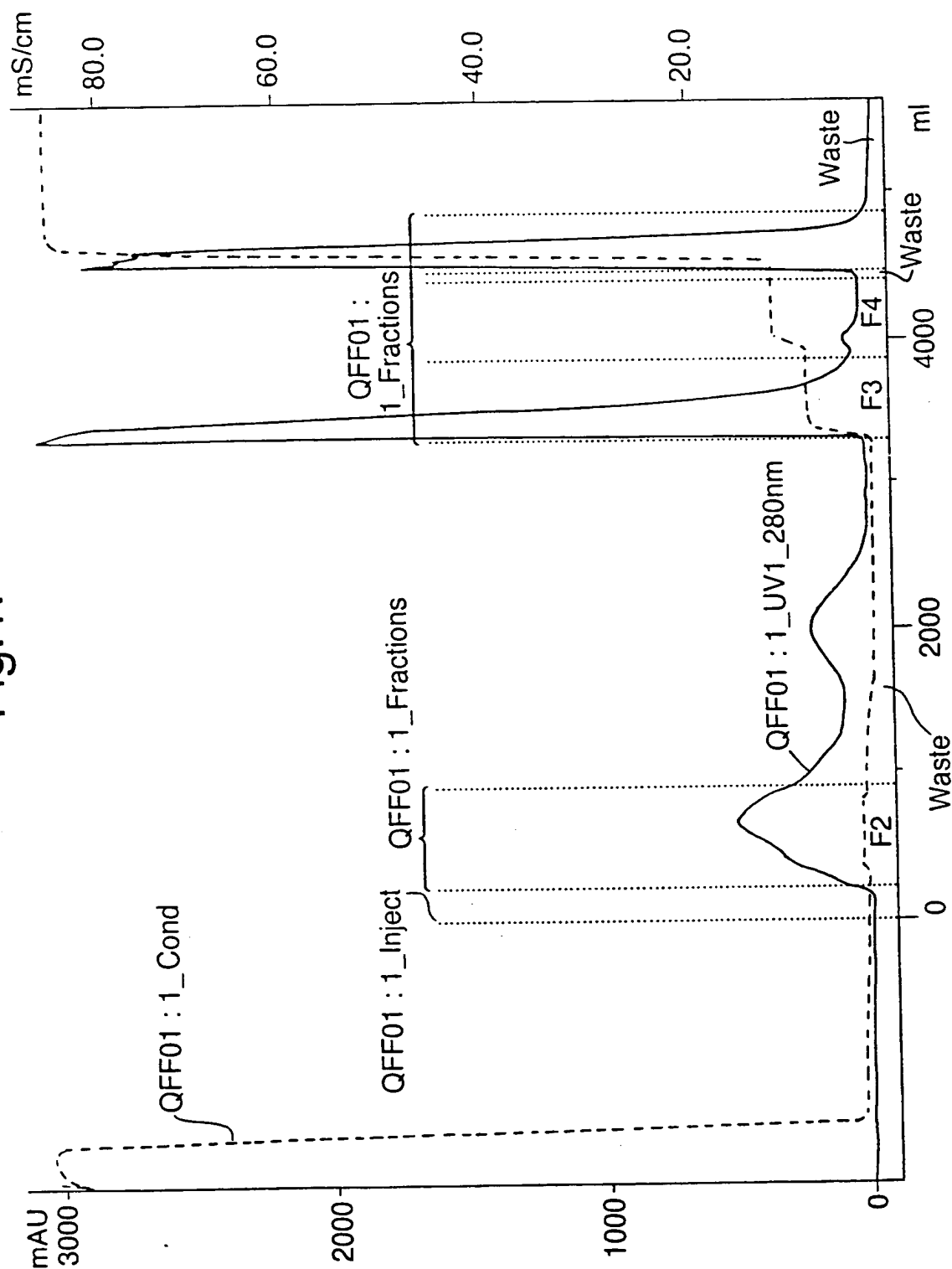
39. Human acid  $\alpha$ -glucosidase substantially as hereinbefore described and with reference to the examples and accompanying drawings.

30



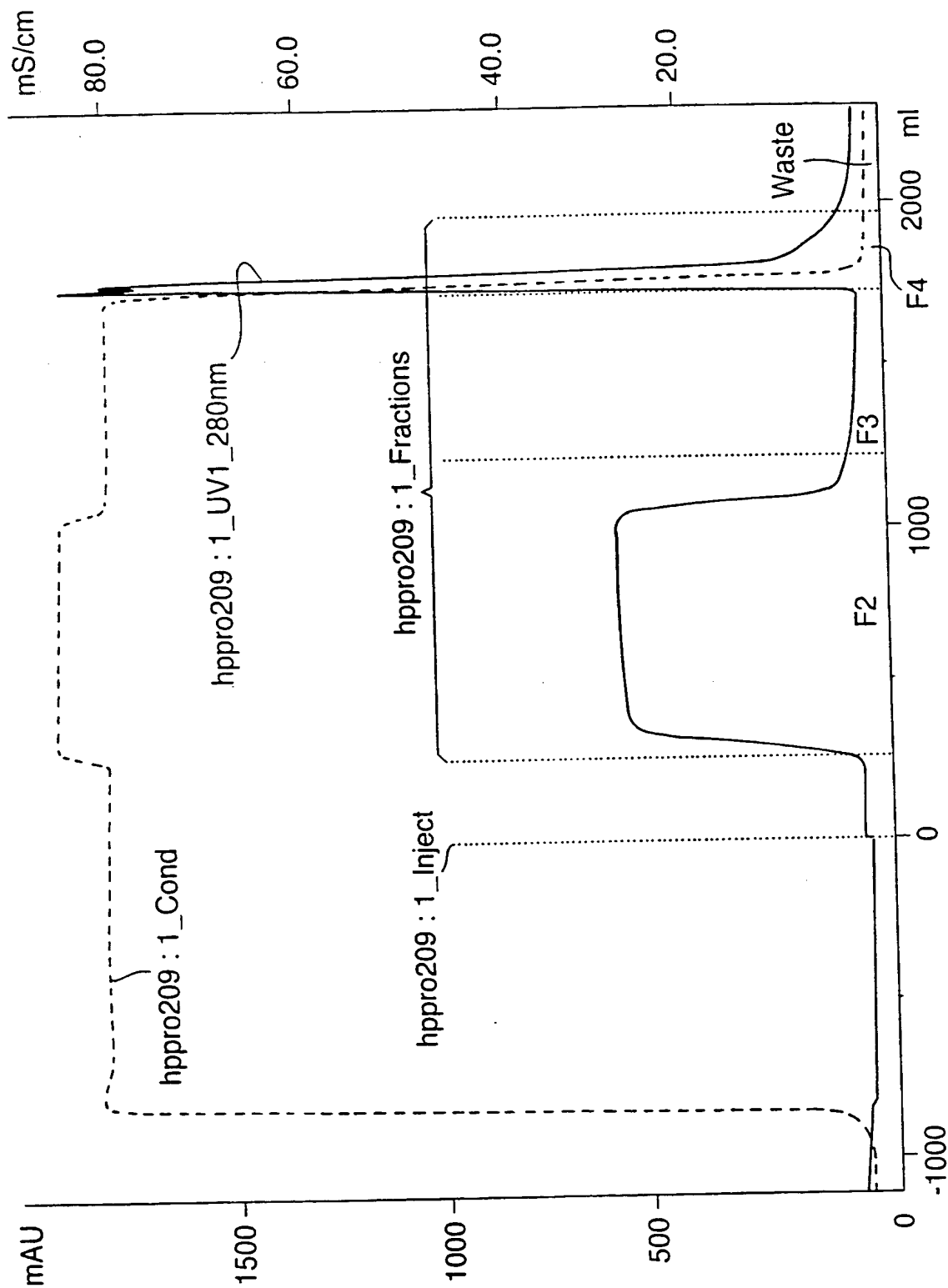
1/22

Fig.1.



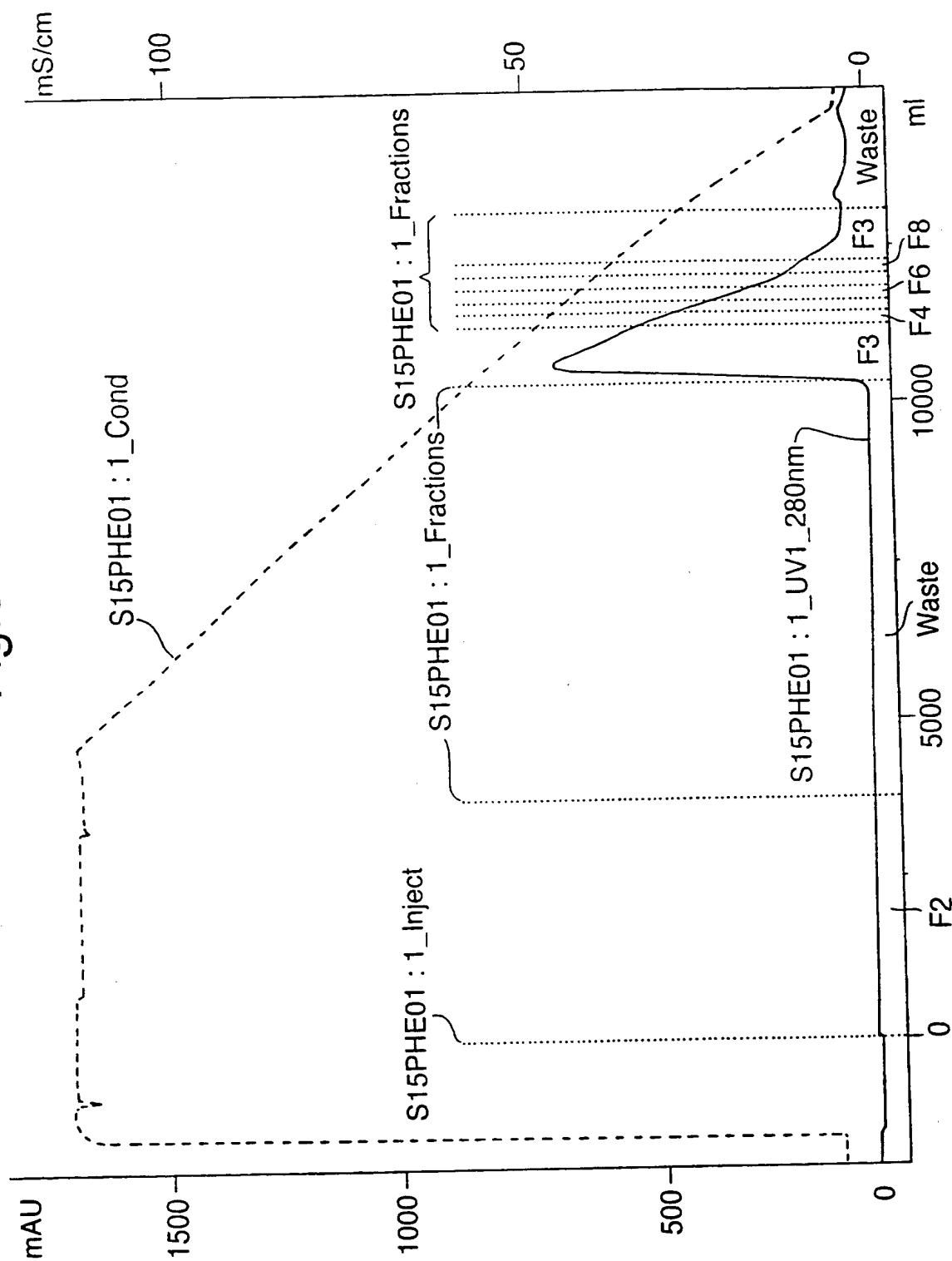
2/22

Fig.2.



3/22

Fig.3.



4/22

Fig.4.

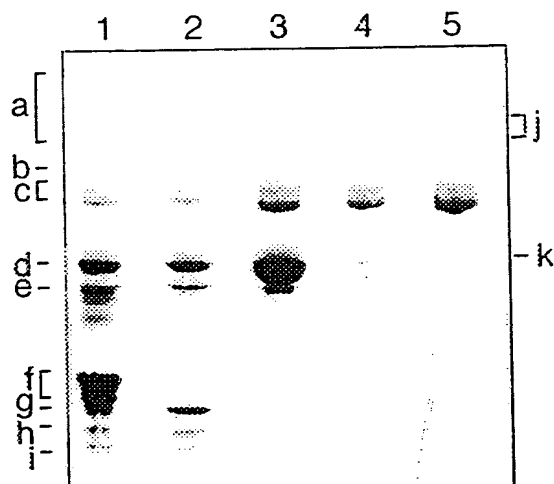
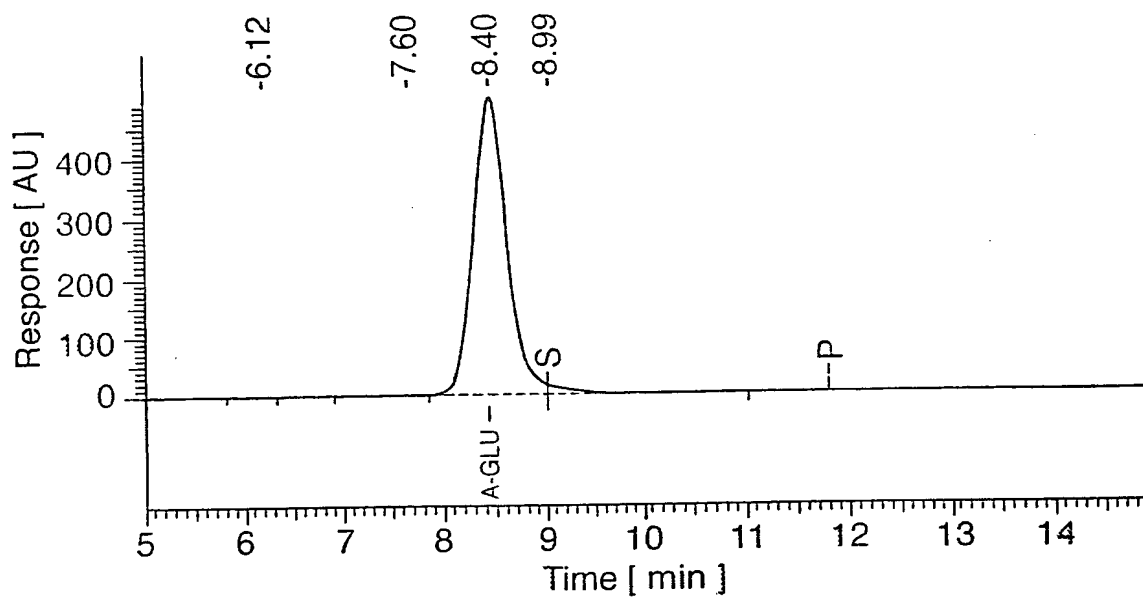


Fig.5.



5/22

Fig.6.

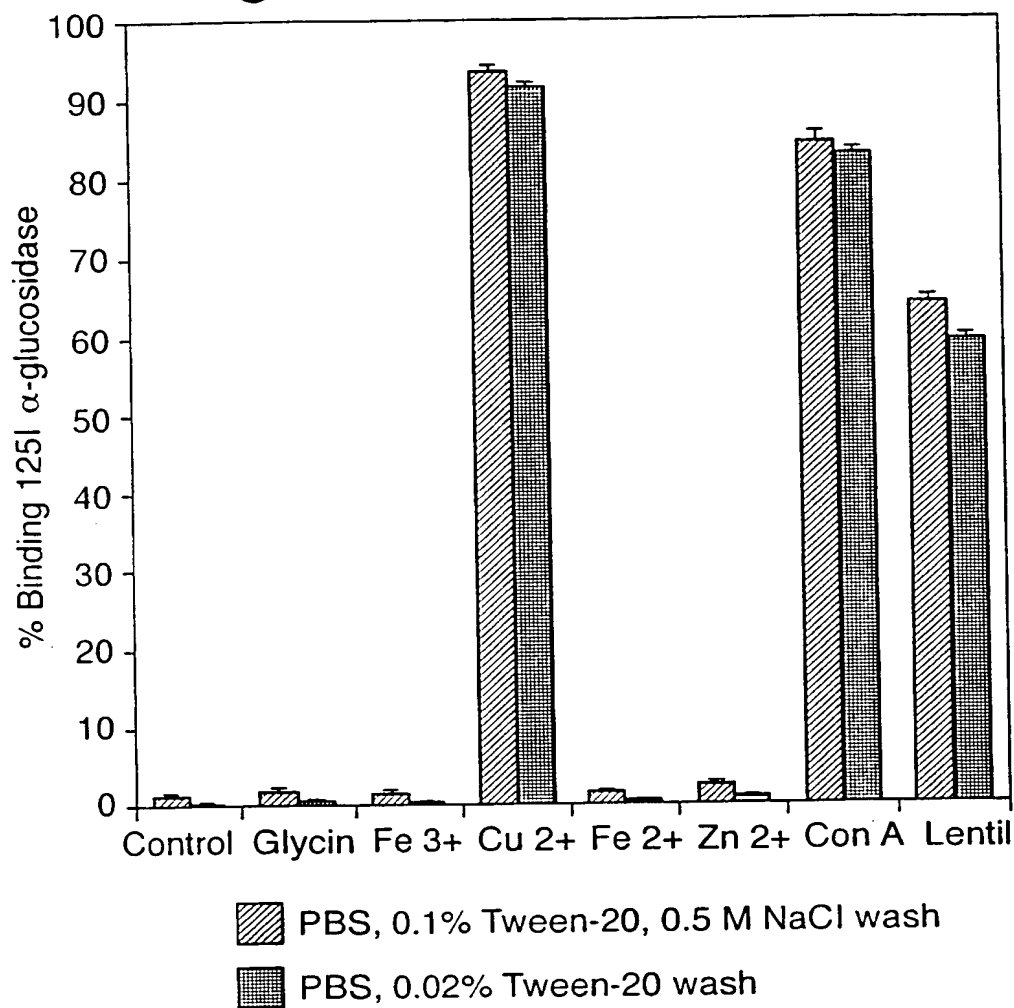
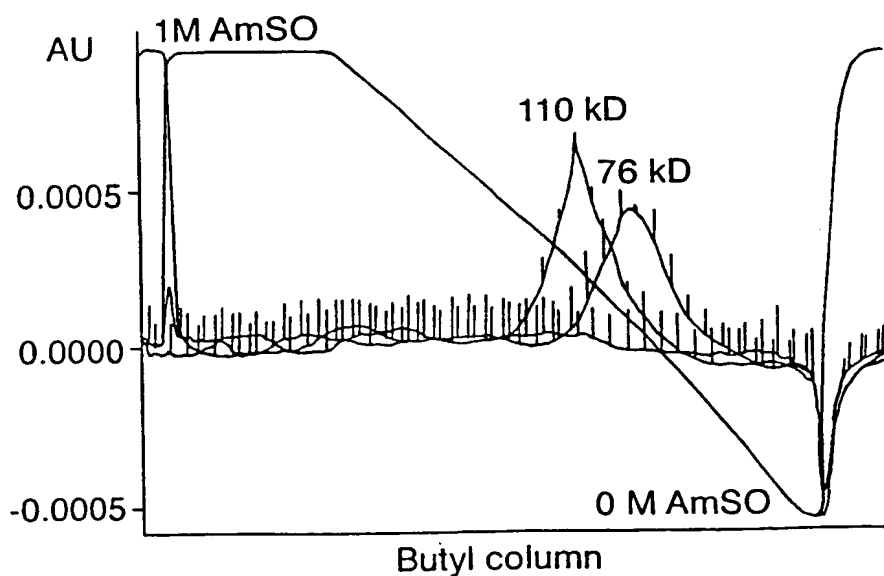


Fig.7A.



6/22

Fig.7B.

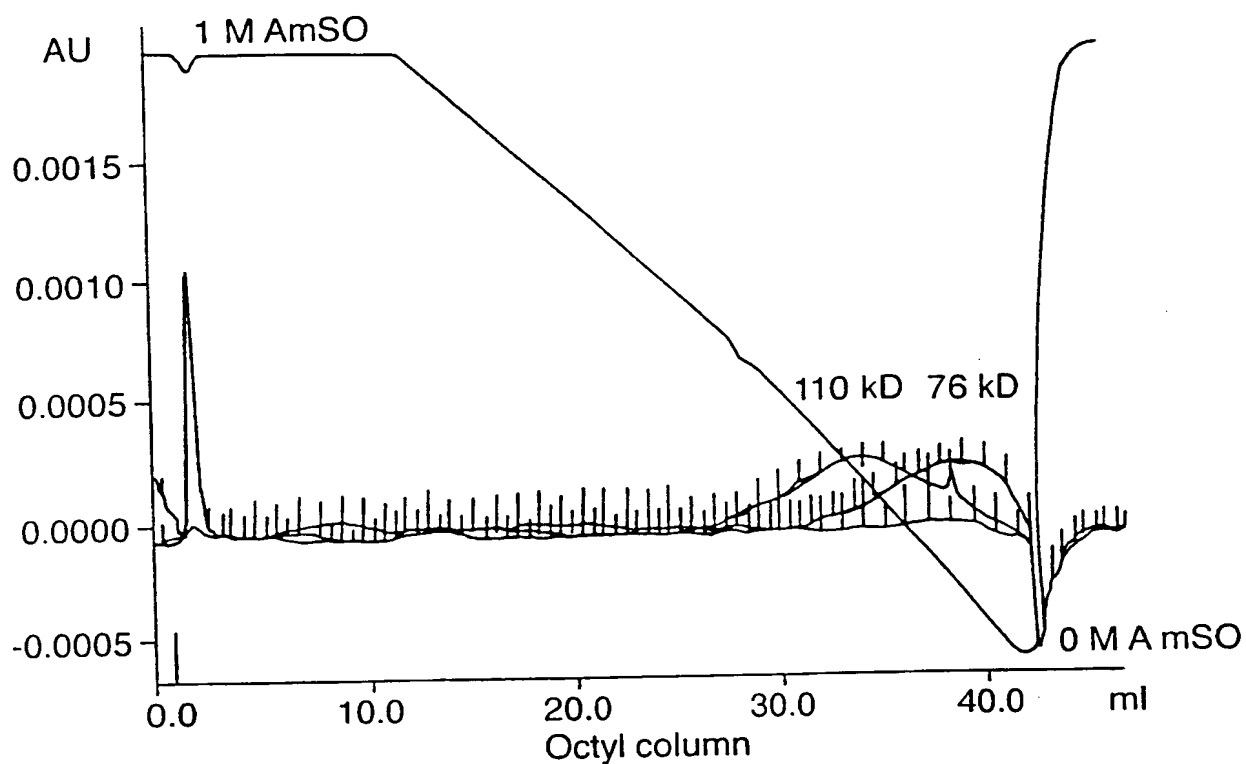
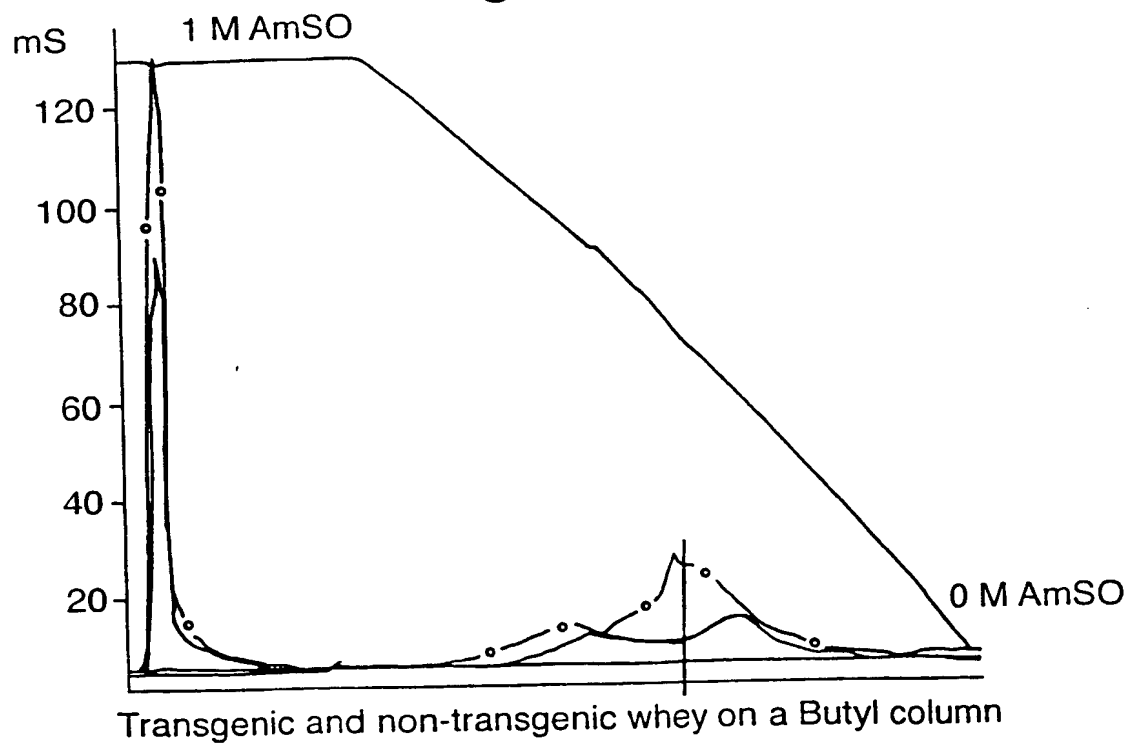


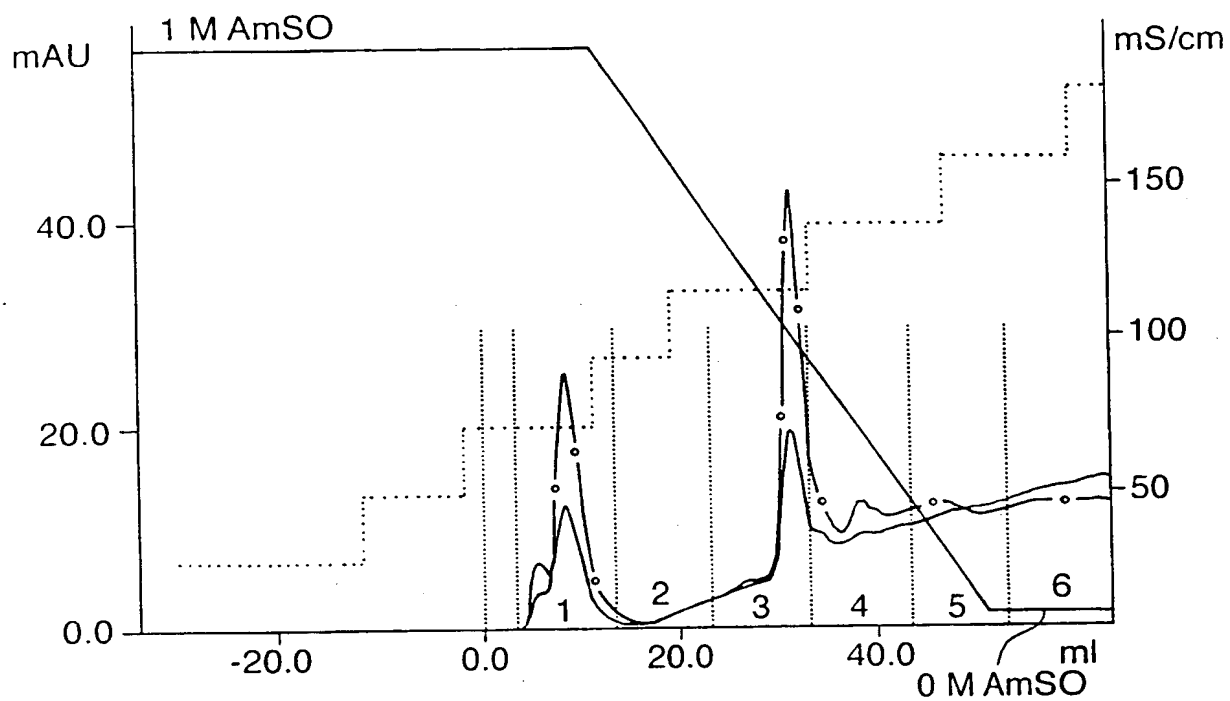
Fig.7C.



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7/22

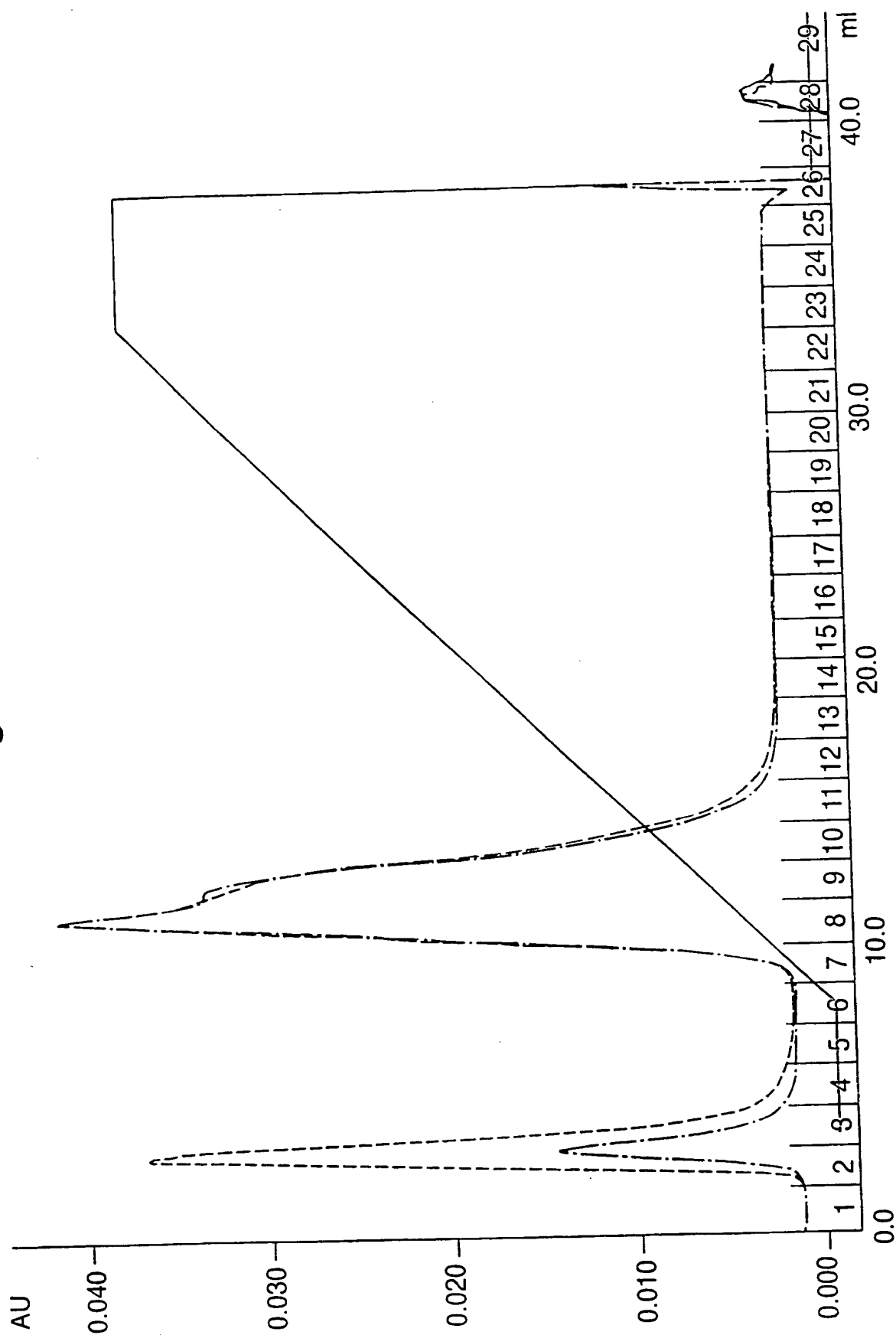
Fig.7D.



SUBSTITUTE SHEET (RULE 26)

8/22

Fig.8.





9/22

Fig.9B.  
non-transgenic whey

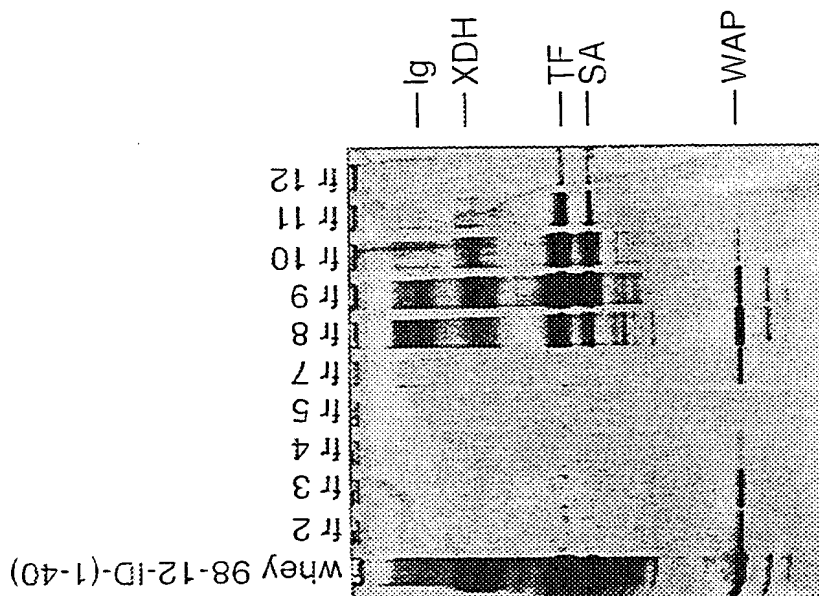
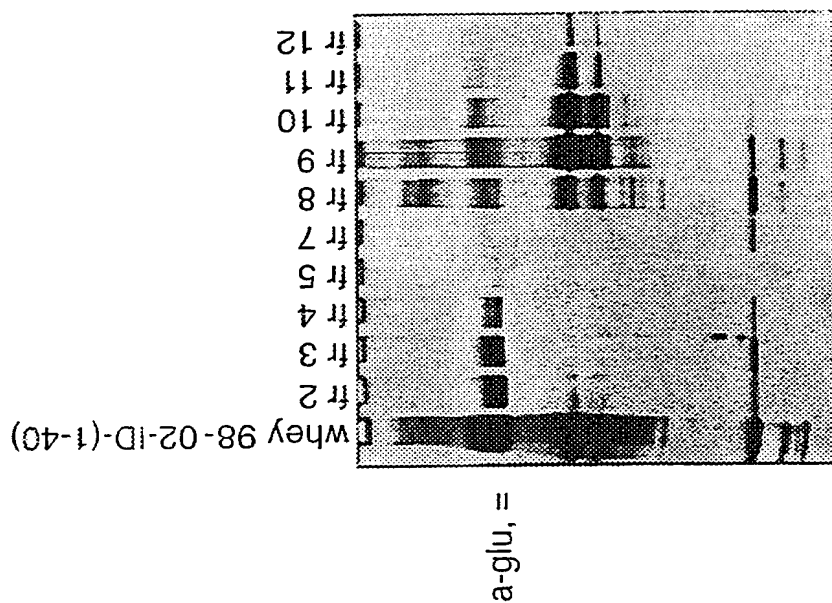
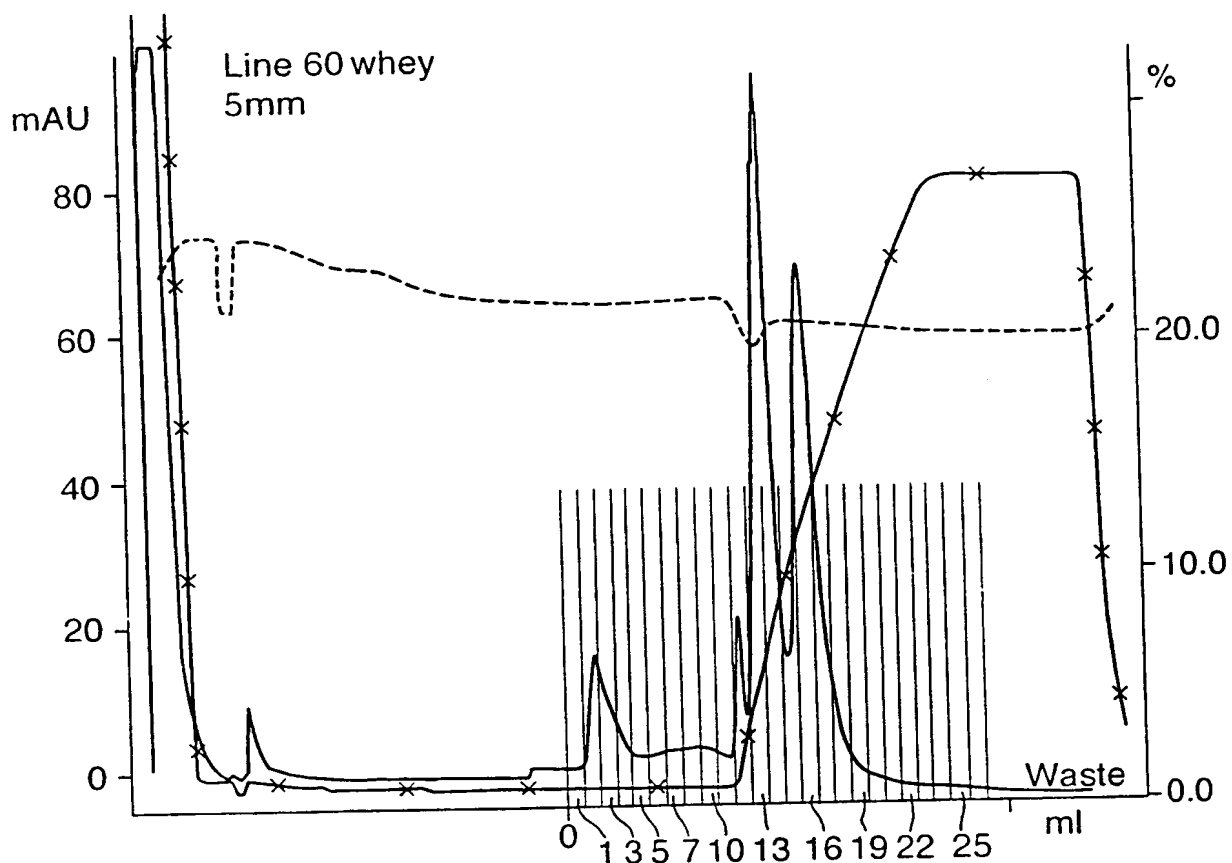


Fig.9A.  
transgenic whey



10/22

Fig.10.



12099801:1\_UV1\_280nm

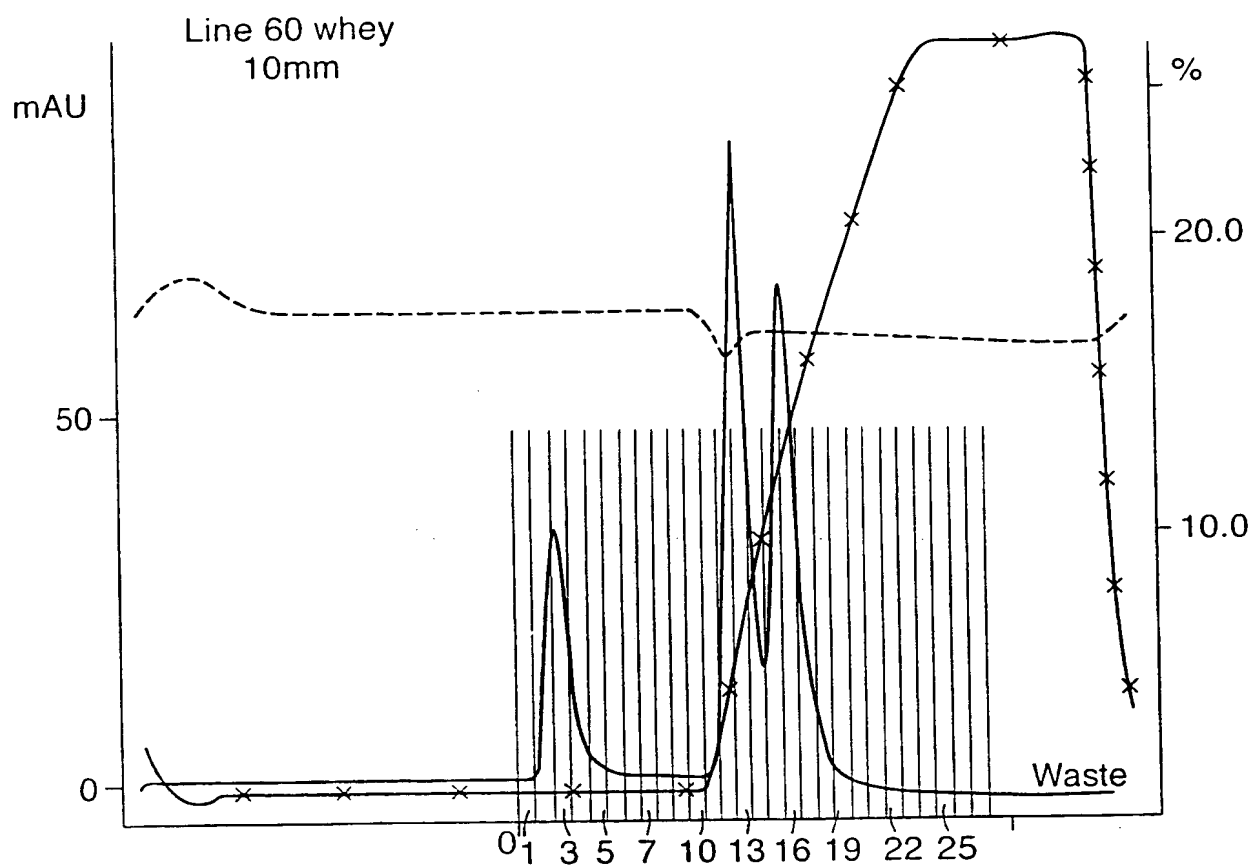
12099801:1\_pH

12099801:1\_Conc%

12099801:1\_Fractions

11/22

Fig.11.



—— 12099802:11\_UV1\_280nm

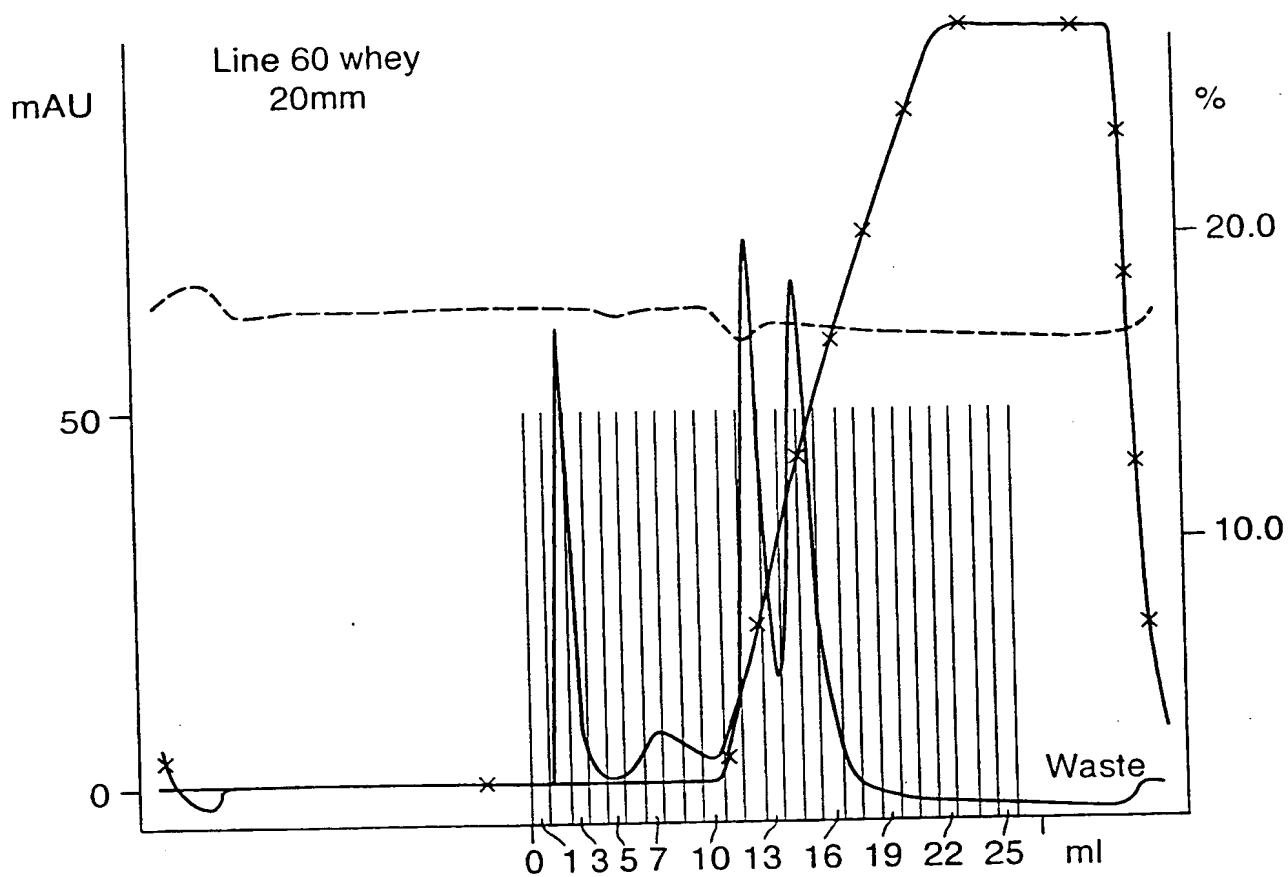
----- 12099802:11\_pH

-x-x-x- 12099802:11\_Cond%

12099802:11\_Fractions

12/22

Fig.12.



—— 12099803:12\_UV1\_280nm

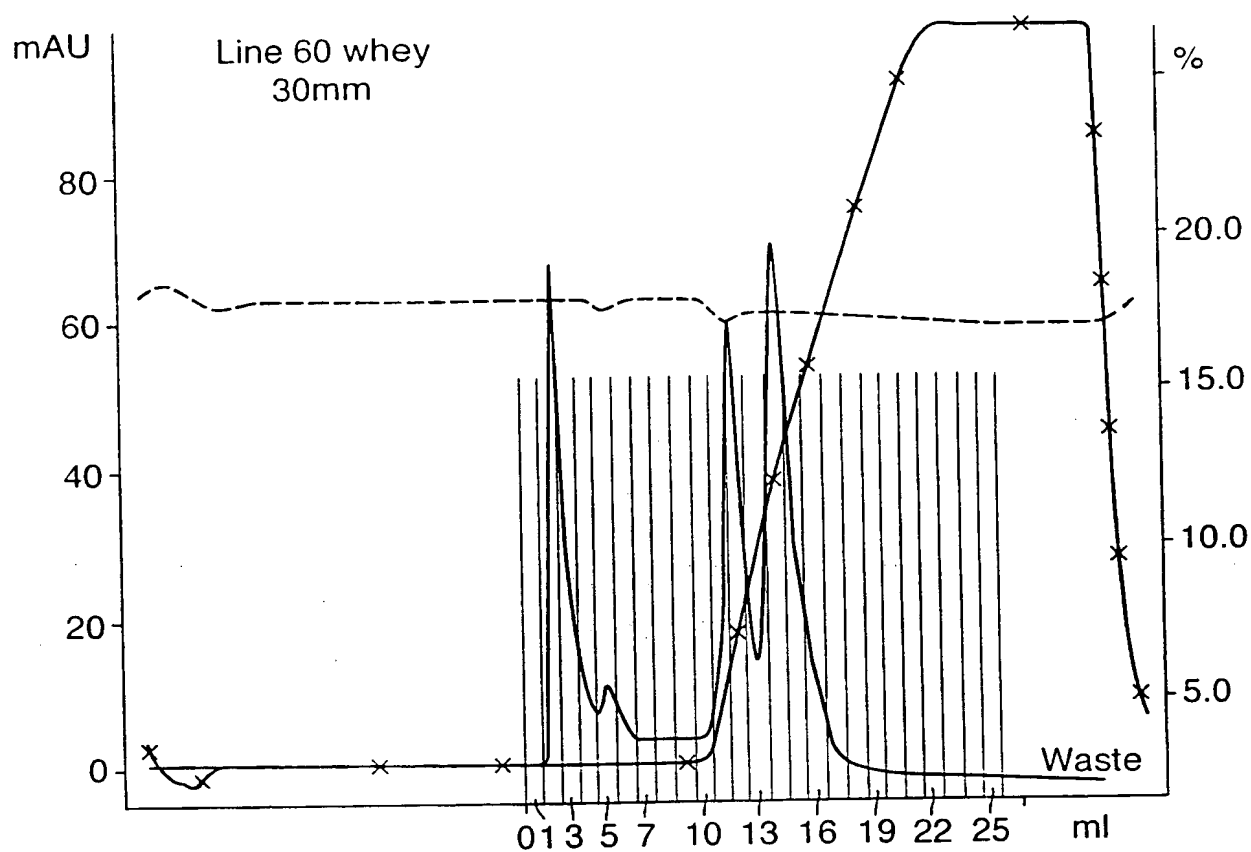
----- 12099803:12\_pH

\*-\*-\* 12099803:12\_Cond%

12099803:12\_Fractions

13/22

Fig.13.



—— 12099804:13\_UV1\_280nm

----- 12099804:13\_pH

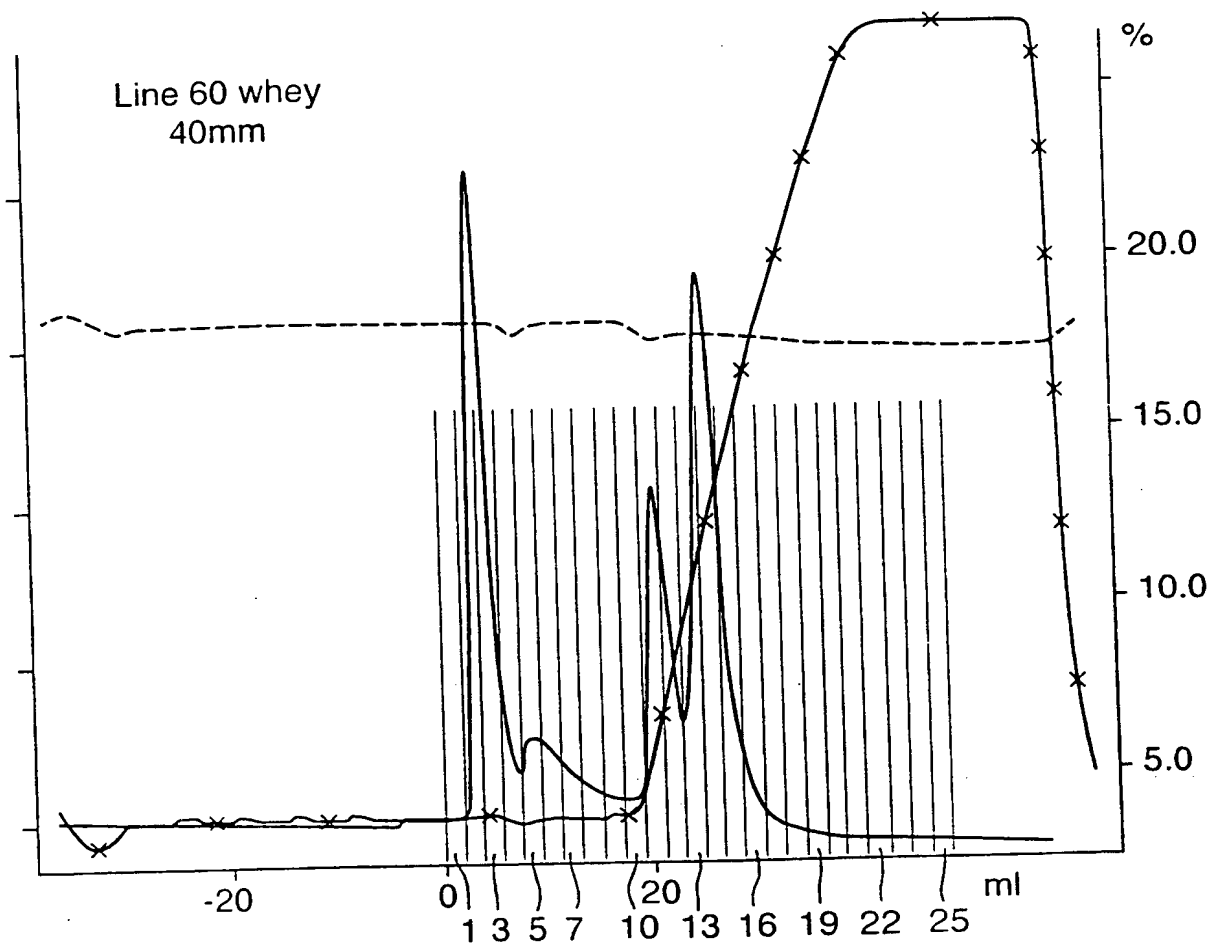
-x-x-x- 12099804:13\_Conc%

12099804:13\_Fractions

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14/22

Fig.14.



—— 121099805:1\_UV1\_280nm

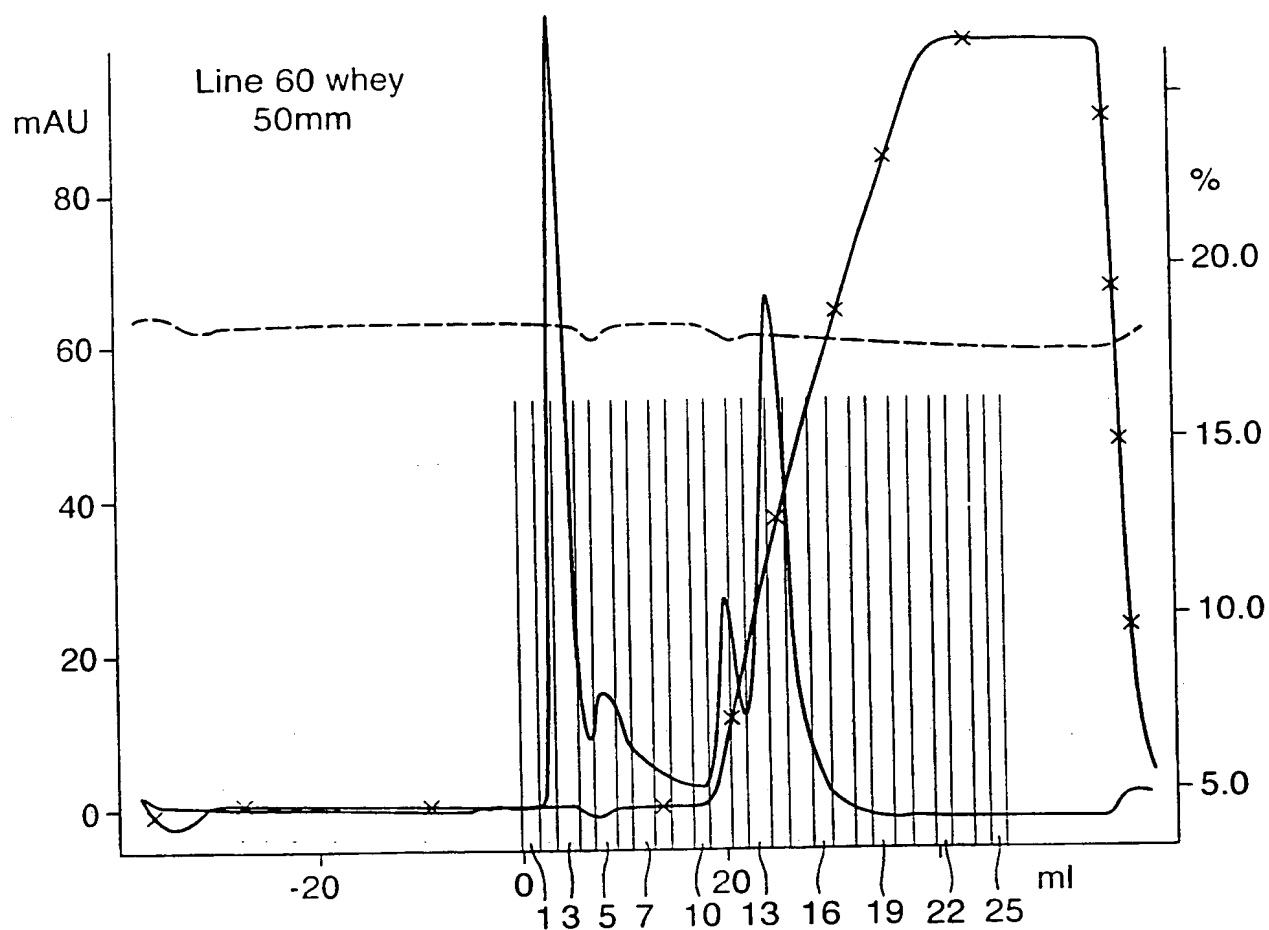
----- 121099805:1\_pH

-x-x-x- 121099805:1\_Cond%

121099805:1\_Fractions

15/22

Fig.15.



—— 121099806:1\_UV1\_280nm

----- 121099806:1\_pH

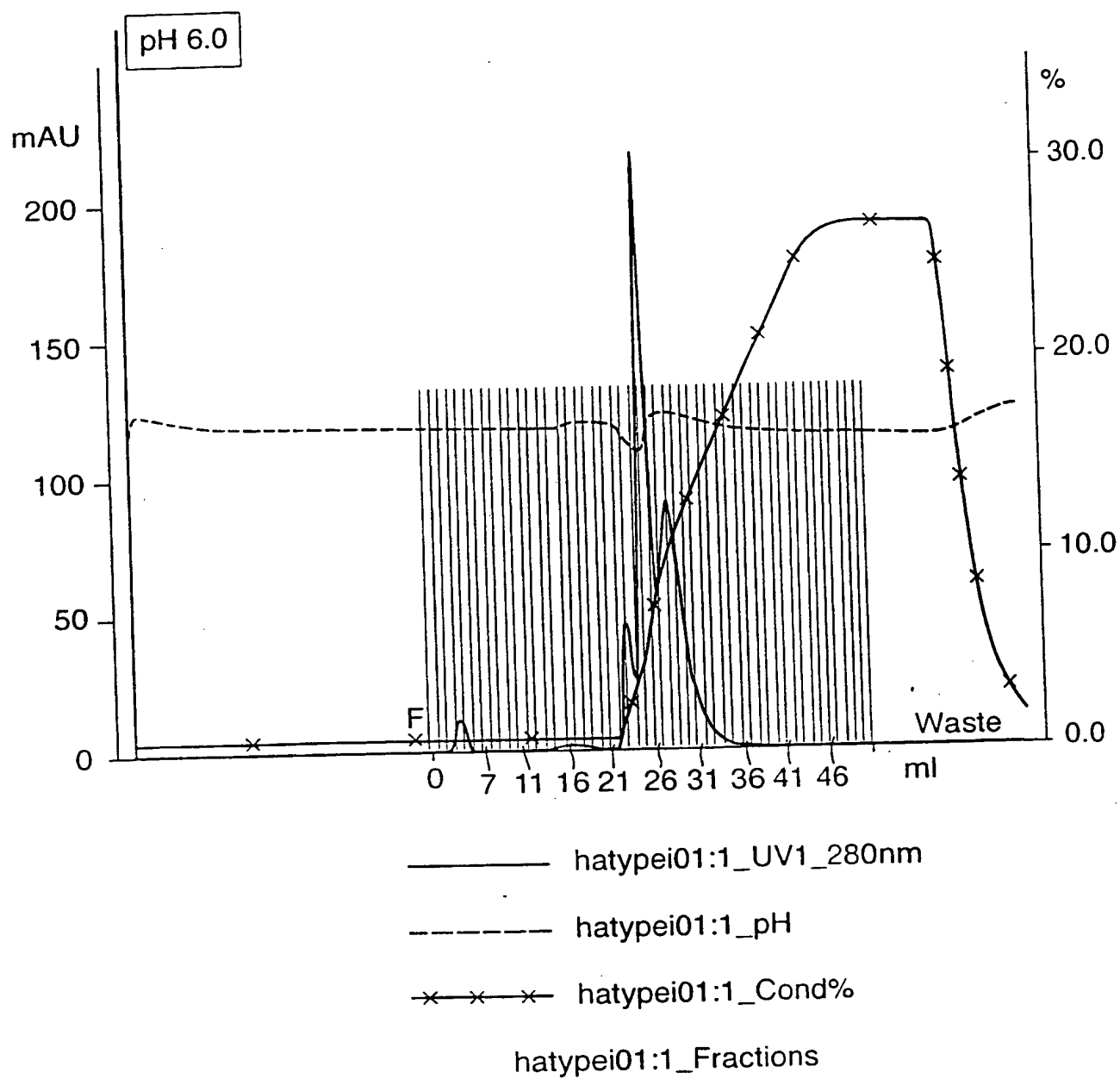
-x-x-x- 121099806:1\_Conc%

121099806:1\_Fractions

SUBSTITUTE SHEET (RULE 26)

16/22

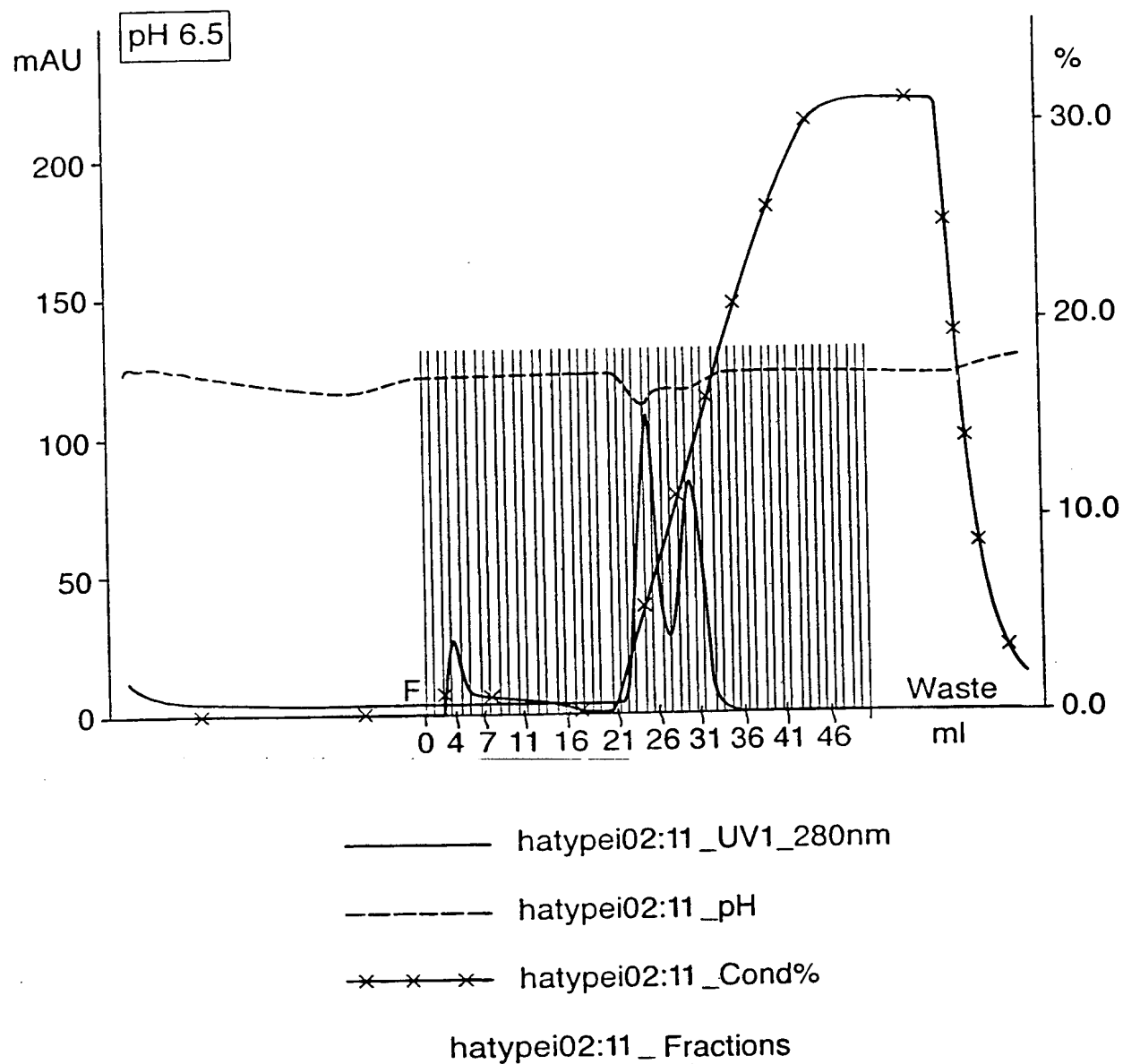
Fig.16.





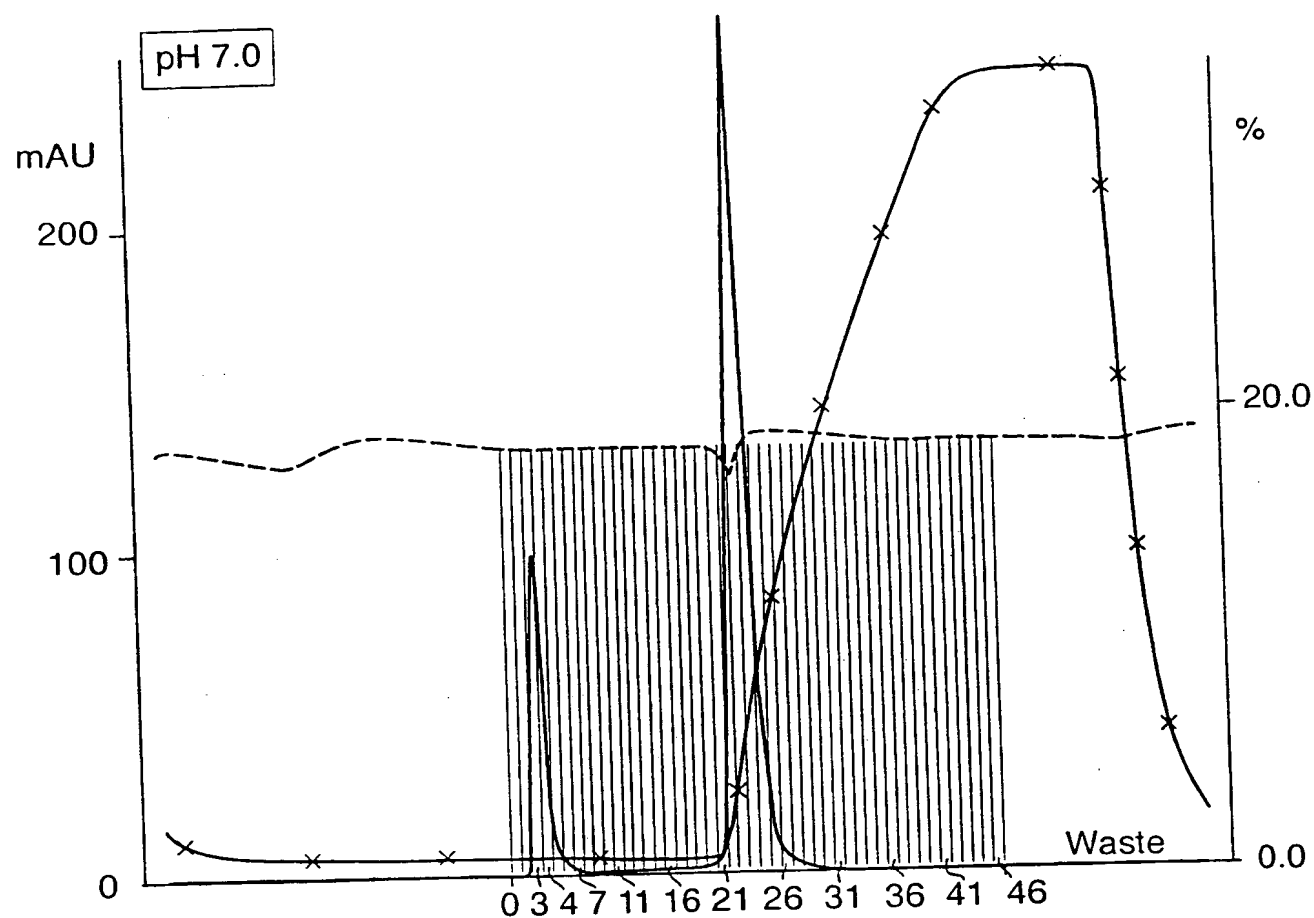
17/22

Fig.17.



18/22

Fig.18.



—— hatypei03:12\_UV1\_280nm

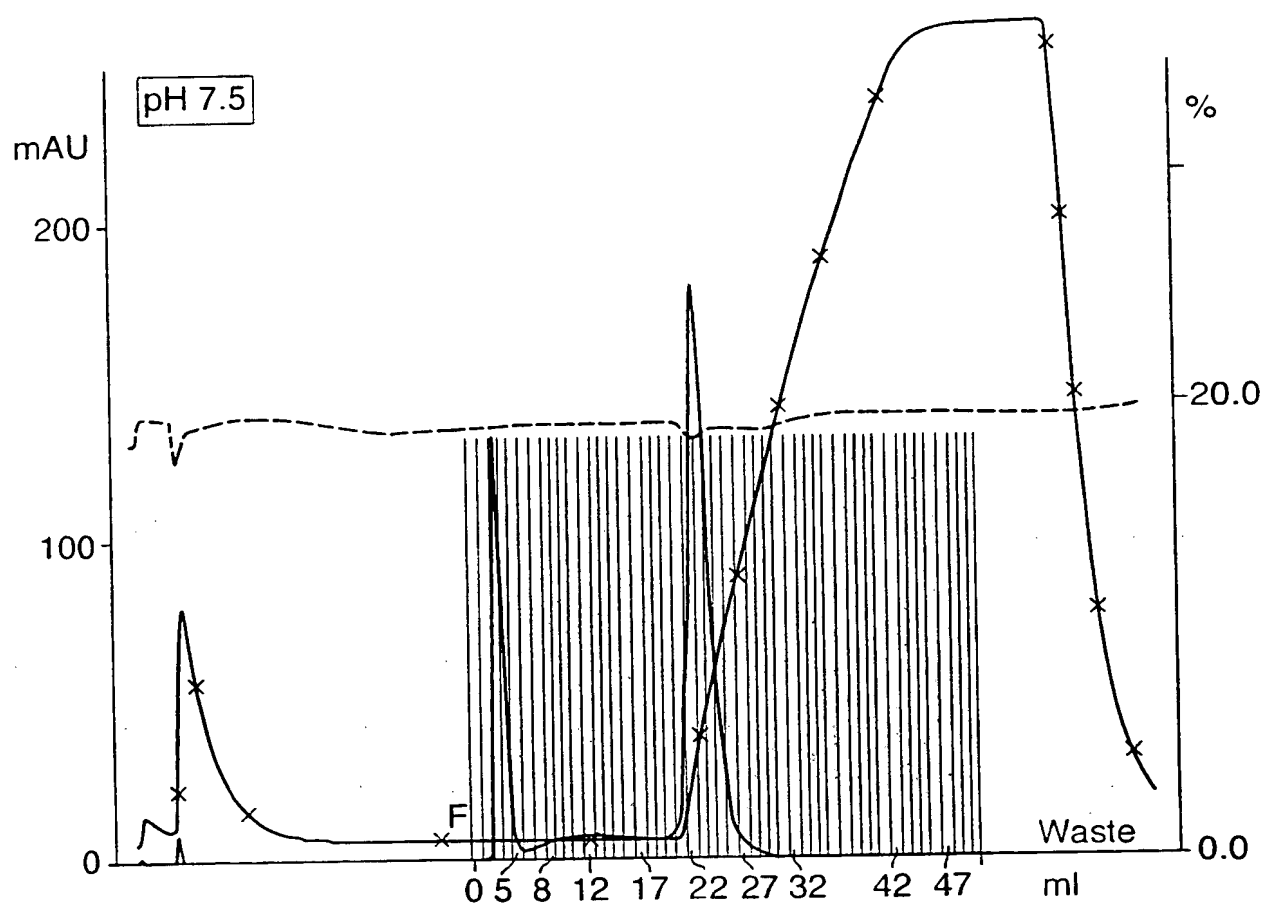
----- hatypei03:12\_pH

-x-x-x- hatypei03:12\_Conc%

hatypei03:12\_Fractions

19/22

Fig.19.



—— hatypei04:13\_UV1\_280nm

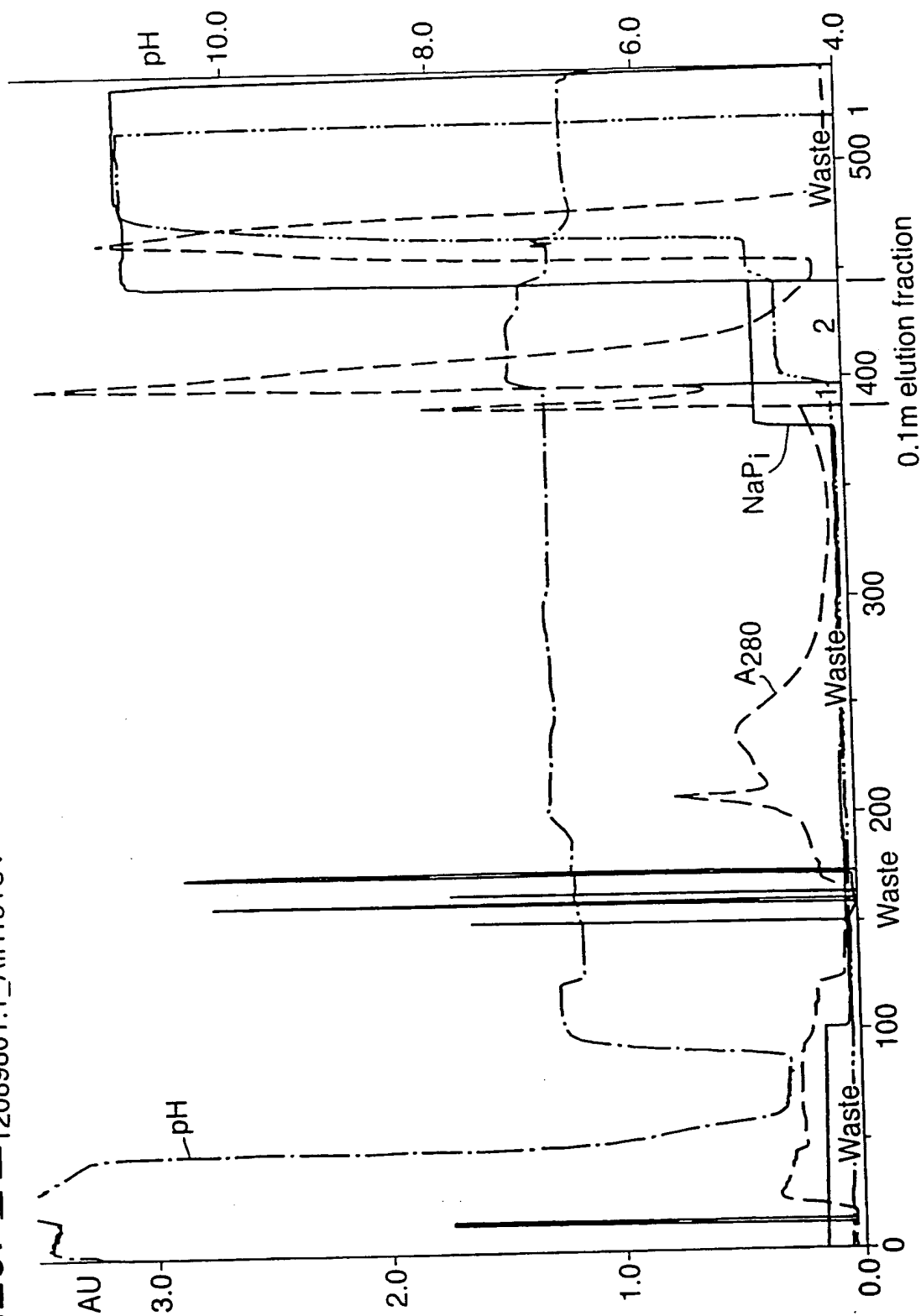
----- hatypei04:13\_pH

-x-x-x- hatypei04:13\_Conc%

hatypei04:13\_Fractions

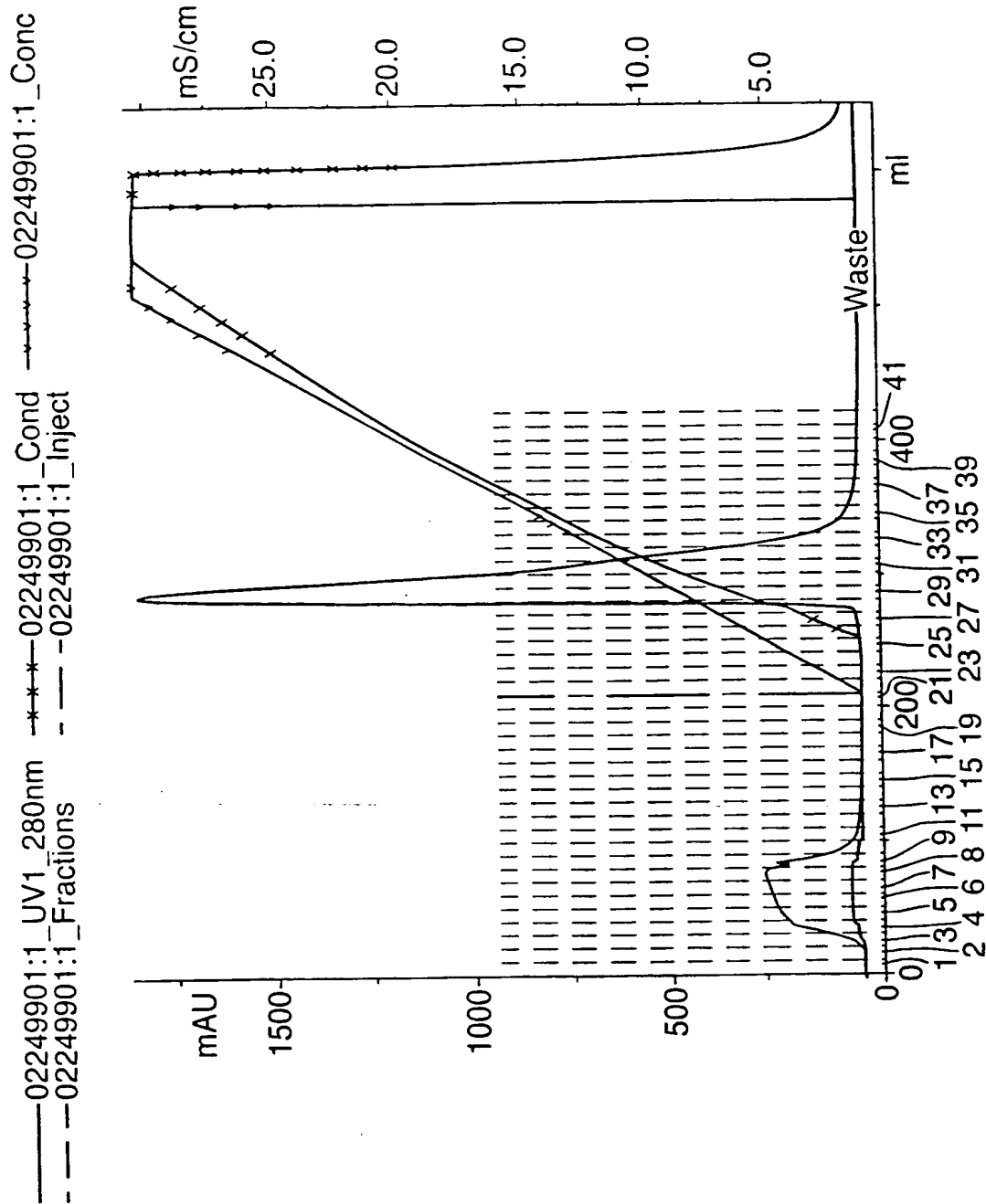
20/22

Fig.20. — 12089801:1\_CIR101 — 12089801:1\_CIR102 — 12089801:1\_AIR121pH  
 - - - 12089801:1\_AIR131UV - - - 12089801:1\_Fractions



21/22

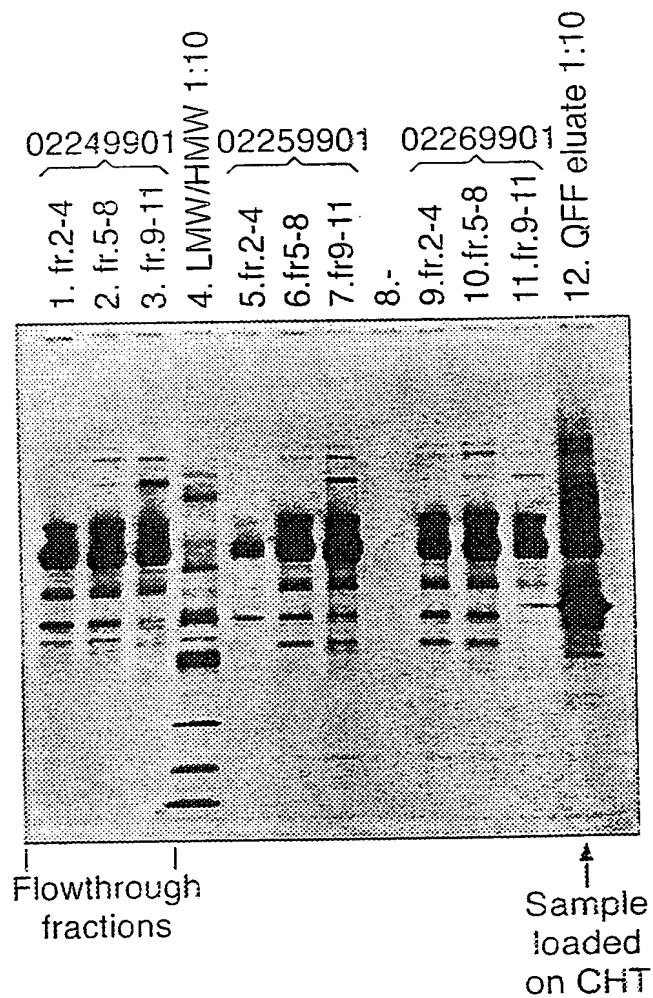
Fig.21.



22/22

## Fig.22.

XK16/15 80°C  
cHT type I 10mM Napi pH 6.5 ; QFF eluate  
Run 02249901/02259901/02269901



SUBSTITUTE SHEET (RULE 26)

# INTERNATIONAL SEARCH REPORT

Internat. Appl. No.

PCT/EP 99/02475

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N9/24 A01K67/027 B01D15/08 C12P19/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIJVOET A G A ET AL: "EXPRESSION OF CDNA-ENCODED HUMAN ACID ALPHA-GLUCOSIDASE IN MILK OF TRANSGENIC MICE" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1308, no. 2, 1 January 1996 (1996-01-01), pages 93-96, XP000609555 the whole document	1, 11, 20-25, 27, 34, 35
A	WO 97 09350 A (PPL THERAPEUTICS SCOTLAND LTD ) 13 March 1997 (1997-03-13)  page 5-13; figure 1  -/--	1-3, 5, 7, 14-16, 21-23



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

12 July 1999

Date of mailing of the international search report

29/07/1999

Name and mailing address of the ISA

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Authorized officer

Mateo Rosell, A.M.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/02475

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WILKINS TD AND VELANDER W: "Isolation of recombinant proteins from milk" JOURNAL OF CELLULAR BIOCHEMISTRY, vol. 49, no. 4, 1992, pages 333-338, XP000611844 the whole document	1,11,16, 29-33, 36,37
A	WO 96 09377 A (AMERICAN NAT RED CROSS ; VIRGINIA TECH INTELL PROP (US)) 28 March 1996 (1996-03-28) abstract page 4, line 12-23 page 15, line 15 - page 16, line 4; claims 8,9	1,11-13, 29,34
A	WO 95 24488 A (ABBOTT LAB) 14 September 1995 (1995-09-14) page 23, paragraph 1 - page 25, paragraph 2; claims 6-8	1,11,12
A	DATABASE WPI Section Ch, Week 8847 Derwent Publications Ltd., London, GB; Class B04, AN 88-333499 XP002108778 & JP 63 245692 A (KANEKAFUCHI CHEM KK), 12 October 1988 (1988-10-12) abstract	1-3,5-7
A	DATABASE WPI Section Ch, Week 9351 Derwent Publications Ltd., London, GB; Class B04, AN 93-408321 XP002108779 & JP 05 304962 A (TAISHO PHARM CO LTD), 19 November 1993 (1993-11-19) abstract	1-3,5
A	DATABASE WPI Section Ch, Week 9650 Derwent Publications Ltd., London, GB; Class B04, AN 96-500360 XP002108780 & JP 08 256770 A (GREEN CROSS CORP), 8 October 1996 (1996-10-08) abstract	1-3
A	EP 0 518 448 A (JOYVALLE NV) 16 December 1992 (1992-12-16) the whole document	5,29,34
A	US 4 981 951 A (TSAY GRACE C) 1 January 1991 (1991-01-01) abstract column 3, line 19-62; example 2	1,6
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## INTERNATIONAL SEARCH REPORT

Internal Application No

PCT/EP 99/02475

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VAN HOVE J L K ET AL., : "Purification of recombinant human precursor acid alpha-glucosidase." BIOCHEMISTRY AND MOLECULAR BIOLOGY INTERNATIONAL, vol. 43, no. 3, 1997, page 613-623 XP002108774 page 615 - page 617 ---	1,2
A	REUSER A J J ET AL., : "DEFECTS IN SYNTHESIS PHOSPHORYLATION AND MATURATION OF ACID ALPHA GLUCOSIDASE IN GLYCOGENOSIS TYPE II" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 260, no. 14, 1985, page 8336-8341 XP002108775 cited in the application page 8337, left-hand column, paragraph 2 ---	1,2
A	FULLER M ET AL: "ISOLATION AND CHARACTERISATION OF A RECOMBINANT, PRECURSOR FORM OF LYSOSOMAL ACID ALPHA-GLUCOSIDASE" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 234, no. 3, 1 December 1995 (1995-12-01), pages 903-909, XP000609446 ISSN: 0014-2956 cited in the application page 904, right-hand column, paragraph 2 ---	1
A	SIENKIEWICZ T. ET AL., : "GROUP SEPARATION OF CASEINS FROM COW MILK ON HYDROXYLAPATITE" NAHRUNG, vol. 32, no. 8, 1988, pages 749-754, XP002108776 page 750 - page 751 ---	1,16
A	S. VISSER ET AL., : "High-performance liquid chromatography of bovine caseins with the application of various stationary phases" MILCHWISSENSCHAFT, vol. 41, no. 9, 1986, pages 559-562, XP002108777 page 559 -----	1,16

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 99/ 02475

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark: Although claim 26**  
is directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful international Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 99/02475

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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US 4981951 A	01-01-1991	NONE	

